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# THE JOURNAL OF PHYSIOLOGY

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## GLYCOGEN AND CALCIFICATION

BY G. E. GLOCK

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THE inhibitory effect of NaF on calcification has been realized for a considerable time, but the exact nature of this inhibition is still unknown. It might be thought that NaF exerts its influence by inhibiting the phosphatase mechanism. There is evidence, however, that NaF does not greatly affect the hydrolysis by alkaline phosphatases, which represent the chief type of phosphatase present in bone and serum, although it inhibits other types of this enzyme. Belfanti, Contardi & Ercoli [1935*a, b, c*] showed that acid phosphatases, which occur in liver, kidney and erythrocytes, are inhibited *in vitro* by NaF, whereas the alkaline phosphatases, found in bone, serum and kidney, are not unless they are in acid media. Robison & Rosenheim [1934] showed previously, using bone slices, that 0.01 *M* NaF did not affect the hydrolytic activity of bone phosphatase, although 0.00001 *M* NaF inhibited the "second" mechanism of calcification. When phosphoric esters were present there was no inhibition.

Kay [1928] found that 0.04-0.08 *M* NaF diminished the rate of synthesis by kidney phosphatase but did not affect the equilibrium. Robison & Rosenheim [1934] think it possible that the "second" mechanism of calcification might involve the synthetic activity of the phosphatase of ossifying cartilage. They suggested that in certain localities in ossifying tissue, the phosphatase is so conditioned, perhaps by water changes at the interface, that its activities are mainly synthetic, so that there is a local transitory increase in the concentration of phosphoric esters, which serve as an increased potential concentration of inorganic phosphate, and, on transfer to another locality of cells or tissue,

where the phosphatase is hydrolytically active, the ester is hydrolysed, finally resulting in the deposition of bone salts.

It is difficult to explain the inhibitory action of very low concentrations of NaF on calcification, and for this reason the present investigation was undertaken, in an attempt to elucidate further the nature of this inhibition.

Laszt & Verzár [1935] described a condition of osteoporosis in young rats resulting from the feeding of Na iodoacetate. Their evidence, however, is unconvincing. Both NaF and Na iodoacetate interfere with the normal carbohydrate cycle of tissues, although at different stages. The effect of NaF on the enzyme systems in yeast and muscle *in vitro* commonly leads to an accumulation of certain phosphoric esters, especially of phosphoglyceric acid. NaF also causes the disappearance of free phosphate from muscle systems and Beattie & Milroy [1925, 1926] found that this was accompanied by the disappearance of glycogen.

Very few experiments have been done on the action of NaF on the metabolism of the intact animal. Lipmann [1930] injected NaF into the lymph sacs of frogs under urethane anaesthesia. In a short time the muscles became practically inexcitable and lactic acid formation was inhibited. The effects are similar to those produced by iodoacetate. In these fluoride-poisoned muscles, there was an increased breakdown of phosphagen and adenylyl-pyrophosphate and marked esterification of hexose with phosphoric acid. These results seem to confirm those obtained with muscle extracts.

A certain amount of work indicating a possible relationship between glycogen and calcification has been reported in the literature. Neumann [1877] showed, by the iodine method, glycogen to be present in all types of cartilage, both foetal and adult. Marchand [1885] stated that "the increase in the size of the cartilage cells in the neighbourhood of the bone is due to an accumulation of glycogen or at least coincides with it". Creighton [1896] found that when teeth of foetal kittens were stained with iodine, the gum epithelium and the dental furrow, but not the enamel organ, contained glycogen. Extending his study to rodents, he showed that glycogen was present in the enamel organ of the incisors (i.e. persistently growing teeth) but not in any others. He also found glycogen in the enlarged epithelial cells of the gum which later form the cement, and found that the disappearance of glycogen from these cells ran parallel with the appearance of calcareous globules. In contrast with the work of Neumann, Creighton found that glycogen was present in all ossifying cartilage, but in no other types. He also found that no glycogen was present in the costal cartilages of rachitic animals. This

investigation was the first to indicate the possible connexion between the occurrence of glycogen and calcification processes. Sundberg [1924] found that ossifying cartilage cells contain more glycogen just before calcification begins and, as it proceeds, the cells disintegrate and the glycogen disappears. He also studied the distribution of glycogen in developing teeth and found it to be abundant in the enamel cord ("Zahnleiste") and in the outer enamel epithelium, but practically never in the inner enamel epithelium and never in the dental papilla. Harris [1932, 1933] demonstrated the presence of glycogen in developing ossifying cartilage of newborn rats and human embryos and found that this disappeared as calcification proceeded. He found that glycogen increased with the age of the cartilage cell, but occurred in hypertrophic cartilage cells only, none being present in the osteoblasts or bone trabeculae. This storage of glycogen in cartilage cells in an area devoid of blood vessels and nerves is according to Harris a "manifestation of its vegetative characteristics". Harris suggested that in calcification there is an association of glycogen, phosphatase, phosphoric esters and vitamin D, the hypertrophic cartilage cell producing both glycogen and phosphatase. (According to Fell & Robison [1930], however, phosphatase is actively secreted by the osteoblasts, and these, according to Harris, are devoid of glycogen.) Harris's view is that the hydrolysis of glycogen leads to the formation of hexose phosphoric esters, and these, under the action of phosphatase and in the presence of Ca ions, would lead to the deposition of bone salts. Robison & Rosenheim [1934] regard blood as the chief source of the phosphoric esters necessary for calcification, but also consider it possible that these might arise from the synthesis or breakdown of glycogen, as suggested by Harris. Harris also found glycogen in developing teeth and observed a gradual transition from a concrete accumulation of glycogen in the epithelium of the dental furrow to discrete particles in the dentine germ and their absence from fully differentiated ameloblasts. Needham [1931] criticizing the early work, wrote: "Nobody now accepts Creighton's views and the attribution of any special embryological importance to glycogen is superfluous. While it may be useful to know the histological distribution of glycogen in the embryo, at present little physico-chemical meaning can be attached to most of this work. Investigators continue to labour along these lines, however." It would seem, however, that the work of Robison and indeed the more recent work of Needham and co-workers on the evocator action of desmo- and lyo-glycogen, does indicate that the distribution of glycogen in areas of future bone formation is of some significance.

Recently, Gendre [1938] studied the occurrence of glycogen in ossifying cartilage of guinea-pig and rat fetuses of different ages. He found glycogen in hypertrophic cartilage cells and osteoblasts. The glycogenic infiltration of the cells was maximal when the medullary vessels were excavating the cavities of the degenerating cartilage cells. He thinks that at this moment the massive arrival of nutritive material to the cells stimulates glycogenic activity. He considers an indirect intervention of glycogen in calcification processes to be probable.

Further evidence in support of the importance of carbohydrate in calcification is afforded by the work of Robison & Rosenheim [1934] that glucose is inhibitory to the "second mechanism".

### METHODS

In the present investigation, a preliminary report of which has already appeared [Glock & Murray, 1938], the glycogen content and the degree of calcification in long bones and in the teeth of rats were followed histologically and also the Ca and glycogen contents of bones were determined chemically. The rats were killed at intervals from 4 days before birth up to about 7 weeks of age. Two groups of rats were used. The diet of each group was that used in previous investigations carried out in this laboratory and consisted of the diet of Coward [1938]. The animals received 15 g. of this stock diet. It was moistened with 15 c.c. water for the control group and for the "fluoride" group with 15 c.c. 0.05% NaF. All the "fluoride" rats used were born of and suckled by mothers who were receiving this fluoride diet and, after weaning, the baby rats also received this diet.

It has been shown with rats by Murray [1936] and Knouff, Edwards, Preston & Kitchin [1936] with dogs, that there is prenatal as well as postnatal maternal transference of fluorine. Murray found that litters of rats born of "fluoride" mothers contained fluorine to the extent of 0.0004% F (= 0.0002 M F), expressed in terms of wet weight, in excess of the control litters. This is considerably greater than the concentration of fluorine which Robison & Rosenheim [1934] found to inhibit calcification *in vitro*, although probably only that part of the fed fluorine which is not deposited in bone could be effective. The metabolic processes of the bones and teeth of the "fluoride" rats used in the present investigation were therefore subjected to the influence of fluorine both pre- and post-natally.

*Chemical investigation.* The long bones of right and left fore- and hindlimbs were removed, scraped well to remove all the adhering muscle and introduced respectively into 4 Pyrex centrifuge tubes graduated at 12 c.c.

and containing 2 c.c. of 60% KOH. (When the bones were larger, 4 c.c. KOH were used.) The tubes were covered and heated in a boiling water-bath for 3 hr., then removed, cooled, made up to 12 c.c. with distilled water and centrifuged. The supernatant fluid contained the glycogen and the centrifugate the insoluble Ca salts. 10 c.c. of the supernatant liquid were removed for the estimation of glycogen, introduced into a 12 c.c. graduated centrifuge tube and evaporated in a water-bath down to 3 c.c. To this were added 1 c.c. 5%  $\text{Na}_2\text{SO}_4$  and 6 c.c. absolute alcohol and the tube replaced in the boiling water-bath until the alcohol just boiled, by which procedure the glycogen and  $\text{Na}_2\text{SO}_4$  precipitates flocculate well. The tube was removed and allowed to stand for 2 hr. and then centrifuged. The residue was stirred up and recentrifuged twice with 65% alcohol and once with 90% alcohol and then dissolved in 5 c.c. warm water. To remove any protein still present, this solution was treated with 2 c.c. Hagedorn and Jensen's  $\text{ZnSO}_4$  solution and 2 c.c.  $N/10$  NaOH, made up to 12 c.c., heated in the boiling water-bath for 3 min., cooled and centrifuged. The glycogen contained in 10 c.c. of the supernatant fluid was hydrolysed by heating for 3 hr. in a boiling water-bath with 2 c.c.  $2N$  HCl. This was cooled, carefully neutralized with solid  $\text{Na}_2\text{CO}_3$ , made up to 12 c.c. and 2, 3, or 5 c.c. portions used for the estimation of glucose by Hagedorn and Jensen's method.

The residue from the original alkali-treated bones was dissolved in warm 10% HCl, made up to 12 c.c. and 1 or 2 c.c. portions used for the estimation of Ca.

*Histological investigations.* The rats were killed by decapitation and the upper and lower jaws and the long bones of forelimbs (humerus, radius and ulna) and hindlimbs (femur and tibia, the fibula being discarded since in most cases it was very small) removed as quickly as possible. The upper and lower jaws were bisected longitudinally and one half of each was placed in Bouin's fixative for subsequent staining for glycogen and the other half in 5% formalin saline to test the degree of calcification. The long bones on one side were hardened for glycogen and on the other for the calcification test, using the same fixatives as for the jaws. Von Kossa's method was used for determining the degree of calcification of the bones and teeth, the sections being subsequently lightly counterstained in safranin. Glycogen was stained by Carleton's [1938] modification of the iodine method of Langhans and then by Best's carmine method. Whenever glycogen was detected by staining, it was confirmed by the fact that after digesting the sections with filtered saliva, glycogen staining reactions were no longer given.

Foetal rats from 3 days before birth up to 7 days old were used for the developing teeth and rats from 5 days before birth up to 14 days old for developing bones. It was found impracticable, owing to the increasing hardness of the teeth and bones, to continue the histological observations beyond this time. Developing teeth were also examined in several foetal kittens of unknown age.

### RESULTS

The results for the Ca and glycogen contents of the bones of the control and fluoride rats are given in Table I and are represented graphically in Fig. 1.

By reference to the graph, it is seen that with the normal rats, the % Ca increases steadily from birth, whereas the glycogen increases rapidly up to about 10 days and then rapidly decreases to remain at an approximately constant level of 0.07 % from the 26th to the 50th day. The results of the "fluoride" rats, however, show that the calcification is inhibited during the first 15 days and after this rises rapidly to the same level as in the control animals. The glycogen is at a much lower level in these "fluoride" animals and reaches a maximum at about 15 days, at which time the degree of calcification suddenly begins to rise. It would therefore seem possible to suggest that the normal progress of calcification in the early stages bears some relationship to the concentration of glycogen in the hypertrophic cartilage cells and that the delay in the calcification observed in the fluoride rats might be attributed to their low glycogen content. That NaF only exerts its inhibitory effect when presented during development is corroborated by other facts. It has been shown in this laboratory that the inhibitory effect of fluoride on the growth of rats is only seen up to about 6 weeks of age, after which time, the growth of "fluoride" rats is similar to that of the control animals. Also, mottled teeth are the result of the intake of fluorine during the period of their development.

Histological examination of the bones showed that those obtained from the foetal rats aged 5 to 4 days before birth, contained glycogen in the cartilage cells and this was principally located in the cells of the region which would later be the primary centre of ossification. At 2 days before birth, when the primary centre of ossification had appeared but calcification had not begun, glycogen was present in the hypertrophic cartilage cells of this region but not in the epiphysial cells. At 1 day before birth calcification had begun in the primary centre of ossification and a small amount of glycogen was present in the epiphysial cells. From 2 to 10 days after birth, when calcification in the shaft was steadily increasing,

TABLE I. Calcium and glycogen content of bones of young rats with and without NaF in the diet.

Age in days	% calcium		% glycogen	
	Control rats	NaF rats	Control rats	NaF rats
1	2.62	—	—	0.087
2	—	3.23	—	—
3	3.23	—	0.40	—
5	—	3.27	—	0.101
6	3.63	—	0.528	—
10	—	3.08	0.740	0.150
15	—	3.14	—	0.248
17	6.41	—	0.24	—
19	—	4.12	—	—
26	7.14	5.88	0.08	0.132
42	—	9.87	—	0.083
49	—	10.18	—	0.053
51	10.36	—	0.073	—

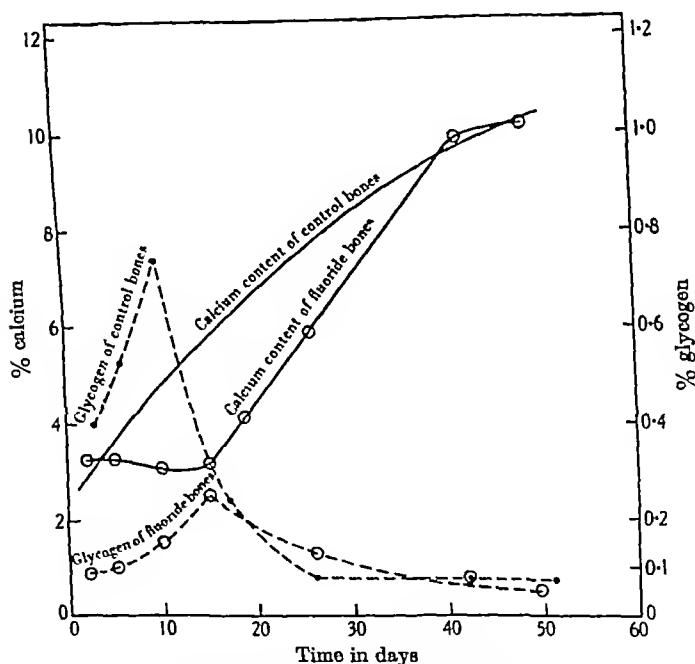


Fig. 1. Curves of the calcium and glycogen contents of the developing bones of control and "fluoride" rats.

and before the secondary centre of ossification had arisen, glycogen was present in both hypertrophic and epiphyseal cells. The glycogen in the epiphyseal cells increased steadily from 1 day before birth to 11 days after birth, after which time it began to decrease. The secondary centre of ossification appeared at approximately 10 days, and calcification



began here at 11 days of age. It would thus seem that the maximal glycogen content of the epiphysial cells corresponded approximately to the beginning of calcification. The glycogen content of the corresponding "fluoride" bones was in all cases less than that of the control rats, as also was the degree of calcification, which calcification in the secondary centre of ossification began 1-2 days later than in the control rats. This observation on the degree of calcification does not coincide exactly with the chemical findings. The percentage Ca in the "fluoride" bones up to 5 days of age was not less than in the controls.

In the jaws, the molar teeth were examined, since in rats these are more comparable to the teeth of man, the incisors of rodents being persistent in growth. In the very early stages of tooth development, such as have been examined in foetal kittens, where the tooth germ, enamel knot and dental lamina are well defined, glycogen was very abundant in the mouth epithelium, lip furrow band, dental lamina and in all the cells of the tooth germ, particularly in the outer limits of the cells of the enamel knot, i.e. the cells which will later give rise to the ameloblasts. At a later stage in development, in the teeth of foetal rats 2 and 1 days before birth, a considerable amount of glycogen was present in the outer enamel epithelium of the enamel organ and less in the stellate reticulum. Definite glycogen granules have not been seen with certainty in the ameloblasts or odontoblasts, and they have never been seen in the pulp. In several sections of rats' teeth, taken 1 day before birth, glycogen was observed in the predentine. In these, the glycogen was not present in the usual form of discrete granules, but this can probably be attributed to the nature of the matrix. Glycogen was found to be absent from the enamel and dentine of teeth after birth. It was still present in the outer enamel epithelium 2 days after birth, but disappeared completely shortly after this. These observations on the distribution of glycogen in developing teeth confirm and extend those of Sundberg and Harris.

Parallel calcification tests indicated that calcification does not commence until the glycogen has practically disappeared. It may be that the first appearance of calcification is coincident with the disappearance of glycogen.

#### DISCUSSION

The results with the bones are more comprehensible if an attempt is made to separate the two stages of calcification, which arise from the primary and secondary centres of ossification respectively. The glycogen and Ca contents of the bones obtained by chemical determination represent the sum of these two stages. Up to 11 days after birth, the increasing

Ca content represents the increasing degree of calcification in the shaft of the bone originating from the primary centre of ossification, whereas at 11 days, when calcification begins in the secondary centre of ossification, and onwards, it represents the sum of the calcification occurring in both shaft and epiphyses. The glycogen contents also represent the sum of the glycogen present in these two regions. From the histological observations, it would appear that the glycogen content of the foetal bones is quite considerable, this increasing up to about 2 days before birth, when calcification begins, and then disappearing except from the rows of hypertrophic cells on either side of the calcifying region of the shaft. From 2 days before birth onwards, the glycogen in these hypertrophic cartilage cells is approximately constant in amount, only increasing as the total number of these cells increases by cell division. The glycogen content of the bones determined chemically, from birth to 10-13 days old, thus represents the sum of an approximately constant fraction present in the hypertrophic cartilage cells of the shaft and a steadily increasing fraction, representing the glycogen in the cartilage cells of the epiphyses. The decrease in the glycogen content after 11 days is caused by the disappearance of the glycogen from the epiphysal cells other than the hypertrophic ones adjoining the calcifying region. It is thus apparent that the cartilage cells of both shaft and epiphyses contain quite a considerable amount of glycogen immediately before calcification begins, but that once calcification has begun the glycogen falls off to a small and more or less constant amount.

It seems feasible to suppose that the glycogen accumulating in the cartilage cells before calcification begins is the initial source of the phosphoric esters. When calcification has begun and the calcifying zone is very vascular, the phosphoric esters of the blood obviously afford another supply. That glycogen serves as the initial source of these esters and the blood as the later source is substantiated by the rapid decrease in the glycogen content of the cartilage cells after calcification has begun. It was noticed, particularly in the 11 and 13 days' old bones, when epiphysal calcification had just begun, that there was a row of very fine glycogen granules at the periphery of the hypertrophic epiphysal cells in addition to the more massive intracellular deposits. Sundberg [1924] found that at this stage the glycogen broke up into very small granules, but did not remark about the peripheral distribution. The presence of these granules in close proximity to the matrix would suggest their participation in some functional activity such as calcification. This would also offer an explanation for the fact that although NaF inhibits the early stages of

calcification, the degree of calcification is eventually as good as the control animals. The lag in calcification observed in the early stages could then be attributed to the low concentration of glycogen, which is then probably the source of the phosphoric esters. The subsequent recovery could be attributed to the fact that the blood, the later source of the phosphoric esters, is probably not significantly different in control and "fluoride" animals.

It is inviting to attempt to correlate the observations on the glycogen content of teeth with the work of Needham and his co-workers. Waddington, Needham, Nowinski & Lemberg [1935] obtained induction of neural tubes in amphibian embryos by implantation of crude glycogen. The significance of the occurrence of glycogen in developing teeth may be that glycogen carries with it an evocator which induces tooth development, and that when all the essential parts of the tooth are present both glycogen and evocator disappear.

It is difficult to say from histological evidence alone whether glycogen breakdown products serve as a source of the phosphoric esters in the early stage of tooth calcification as the results indicate it does for bone. There seems, however, no reason why glycogen should not play the same role in both, firstly as a possible initiator of development and secondly as the primary source of the phosphoric esters required for calcification.

#### SUMMARY

1. The glycogen content and the degree of calcification of developing long bones and teeth of foetal and young rats were studied histologically and in addition chemical determinations of the Ca and glycogen contents of the bones were made.

2. These observations were carried out on normal rats, and on rats suckled by mothers receiving 0.05% NaF in their diet, and subsequently themselves receiving this diet.

3. The histological observations in the case of bones were amply confirmed by the chemical determinations.

4. The glycogen content of the developing bones determined chemically, increased up to about 10-13 days after birth and then rapidly declined, whereas the calcification increased progressively.

5. The glycogen content of the bones of the "fluoride" rats was considerably less at all ages than the corresponding control rats and the maximal concentration, which was only a third of that of the control animals, was reached later. Calcification was retarded in the early stages of development, but eventually reached the same level as the controls.

6. Glycogen was only present in very early stages of tooth development and disappeared as soon as or soon after calcification began.

7. It is suggested that in both tooth and bone development, glycogen might first initiate the differentiation and later serve as a primary source of the phosphoric esters required for calcification.

8. The inhibitory effect of NaF on bone calcification might be attributed to the low glycogen content which resulted from the administration of fluorine.

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THE EFFECT OF TEMPERATURE ON  
THE MECHANICAL RESPONSE AND THE  
VISCOSITY AND OXYGEN CONSUMPTION  
OF UNSTRIATED MUSCLE

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THE effect of temperature on unstriated muscle has been described by several workers [Schultz, 1897; Eckstein, 1920; Winton, 1927], but since it was found that the two excitabilities in unstriated muscle are affected differently by various agencies [Singh, 1938*b*], it was deemed expedient to determine the effect of temperature, as this also does not affect them identically. As temperature may affect the viscosity of the muscle [Gasser & Hill, 1924], certain experiments were performed to elucidate this probable action and, as viscosity has been considered to play an important part in enabling unstriated muscle to maintain a tonic contraction without expenditure of energy [Bayliss, 1928; Winton, 1937], the oxygen consumption of the muscle under various experimental conditions was also recorded.

METHODS

The oxygen consumption was measured in Warburg manometers, which were shaken in an "Aminco" thermostat maintained electrically at  $27 \pm 0.05^\circ \text{C}$ ., or at any other desired temperature by means of electrical heating combined with cooling with a refrigerating unit. Manometric vessels of 15-17 c.c. capacity with single side bulbs were used.

The  $Q_{O_2}$  of the muscle in saline varies from frog to frog and hence, in experiments involving comparison of oxygen consumption of control and treated muscles, the frog stomach was divided into two nearly equal halves, one of which was used as the control and the other as the experimental. The muscle was mounted on a glass frame which consisted of a thin glass rod bent at right angles at two places to form a double L, the middle limb of which was 2 cm. and the ends about 0.75 cm. long;

the muscle was slightly stretched, and the ends tied to the short limbs, but, if the muscle was rather long, it was folded transversely and fixed at one end of the frame and the two ends were tied to the same limb. The frame was accommodated in the flat bottom of the vessel. The side bulb contained 0.1 c.c. of 20% KOH and the volume of the fluid was 4 c.c. At the end of the experiment the muscle was dried to a constant weight in an oven and weighed.

For a.c. stimulation, a vessel of 40 c.c. capacity was used, two silver electrodes were fixed by means of enamelled copper wires passing through a tightly fitting rubber stopper in a side opening, and the muscles were placed lengthwise between the electrodes. If adrenaline was used in any solution, a control flask was set up to record any oxygen uptake by the drug itself. In these experiments the tension produced could not be recorded but a control muscle was set up outside under similar experimental conditions and its behaviour recorded.

## RESULTS

*Effect of temperature on the response to ions outside.* Tone is affected by changes of temperature in a curious way (Fig. 1). In frog and *Mytilus*

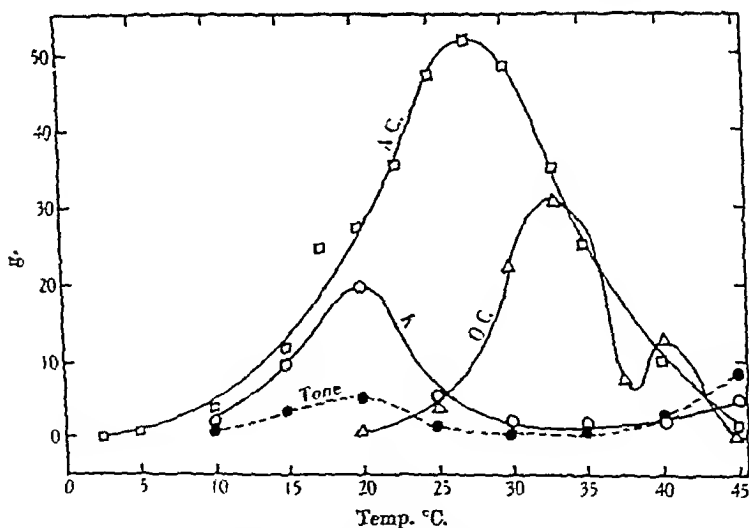


Fig. 1. The effect of temperature on the response of frog muscle to a.c. (10 V./10 sec.), potassium (0.04 M KCl), on the a.c. off-contraction and tone.

muscles tone increases with temperature up to 20° C.; then it declines up to 35–40° C. and increases again up to 45–46° C. Further increase

in temperature produces a rapid and irreversible loss of tone; the muscle becomes inexcitable to all forms of stimulation. The decline in tone above  $20^{\circ}\text{C.}$  and the secondary rise at high temperatures are sometimes absent.

The contracture produced by sodium salts, bromide, nitrate, iodide, thiocyanate (the chloride of the saline being replaced with the anion) is affected similarly. As with normal tone, there may, in thiocyanate, be a continuous increase in contracture with temperature.

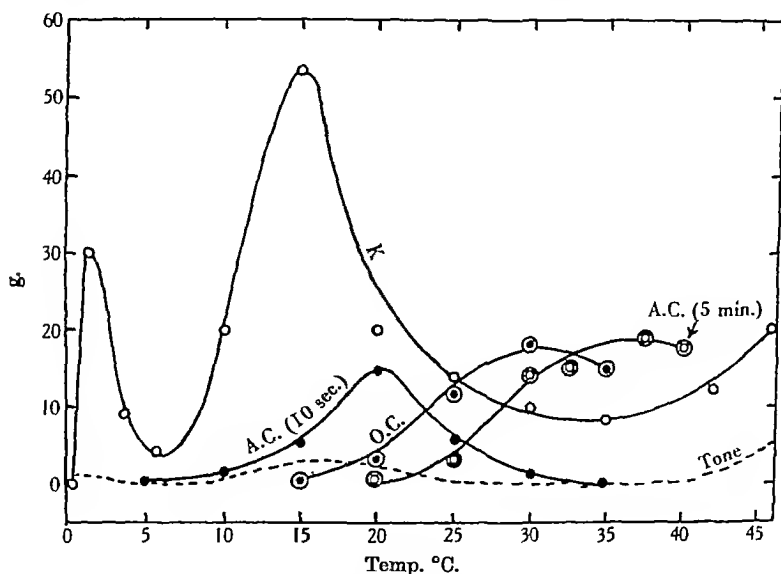


Fig. 2. The effect of temperature on the response of *Mytilus* muscle to A.C. (10 V./10 sec.) and A.C. (10 V./5 min.), potassium (0.1 M KCl), on the A.C. off-contraction and tone.

In frog muscle the optimum temperature for the potassium contracture is also  $20^{\circ}\text{C.}$ ; the secondary rise is either absent or slight. In *Mytilus* muscle the excitability to potassium is affected more or less similarly (Fig. 2), the optimum temperature being  $14-15^{\circ}\text{C.}$ ; a difference between frog and *Mytilus* muscle is that the latter may exhibit increased excitability at low temperatures ( $2-3^{\circ}\text{C.}$ ). In frog muscle the A.C. off-contraction increases with temperature up to  $33-35^{\circ}\text{C.}$ , and in *Mytilus* muscle up to  $35-37^{\circ}\text{C.}$  These observations may be accounted for by assuming that the stimulating power of ions outside, especially the anions, increases uniformly with temperature ( $20-45^{\circ}\text{C.}$ ); but that adaptation also increases with temperature, so that there is an intermediate decrease in excitability. That this is likely is shown by the following experiments: (1) In *Mytilus* muscle adaptation to potassium

increases so much that the contraction resembles an A.C. twitch at about 30–35° C. (2) This decrease may be absent in thiocyanate which decreases adaptation. (3) The secondary rise is slight or absent in excess of potassium to which adaptation is rapid. (4) It is absent with the A.C. off-contraction obtained by passage of A.C. for 3–5 min. With such a procedure the process of adaptation is antagonized by ions outside, with the result that the A.C. off-contraction and the tension at the end of the period of prolonged stimulation increases with temperature up to 35–37° C.

*Influence of temperature on the response to A.C.* The response to A.C. increases with temperature up to a certain value and then declines; the optimum temperature in frog muscle varies from 20 to 30° C., possibly depending somewhat upon the composition of the saline (Table I).

TABLE I. The optimum temperature for A.C. (10 V./10 sec.) in frog stomach muscle.

No. of exp.	In frog saline	pH	Osmotic pressure, times normal	Optimum temperature ° C.
1	Normal	7	0.92	27
2	Normal	7	1.0	30
3	Normal	8	1.0	20–25
4	0.027 M KCl	8	0.92	25–30
5	0.027 M KCl	8	1	30
6	Cl replaced with NaNO <sub>3</sub>	8	0.92	25
7	Cl replaced with iodide	7	1.0	25
8	Cl replaced with iodide	8	1.0	30
9	Cl replaced with SCN	8	0.92	30
10	Cl replaced with SCN	8	1	25–30

These variations in frog muscle can be understood by studying the effect of temperature on *Mytilus* muscle. The optimum temperature for A.C. in *Mytilus* muscle depends upon the duration of the stimulus; for A.C. for 10–60 sec. it is 20° C., and for A.C. for 3–5 min. it is 35–37° C. If A.C. is passed for 10 sec. the optimum temperature is less than in frog muscle, owing probably to the greater sodium chloride content of *Mytilus* saline; but with longer duration of the passage of the current adaptation comes into play and the optimum temperature increases. In frog muscle adaptation to A.C. increases with temperature, and in *Mytilus* muscle it decreases; this difference is due to the greater sodium chloride content of *Mytilus* saline.

The optimum temperature for the response of frog muscle to acetylcholine is 30° C. and that for the ammonium withdrawal contraction is the same as that for the A.C. contraction; the optimum temperature for the ammonium contraction is, however, the same as that for the potassium contraction.



*Viscosity*

The response of unstriated muscle is more sluggish than that of striated muscle and this has been ascribed to the greater viscosity of the former [Hill, 1926]; the relaxation of unstriated muscle is particularly slow, and the tension which the muscle thus maintains for a considerable period has also been ascribed to increase in the viscosity [Bayliss, 1928; Winton, 1937]. During the present and previous experiments it was noticed that sometimes the responses of plain muscle were brisk, and at other times sluggish, and in the present experiments it has been possible to vary the sluggishness of the response.

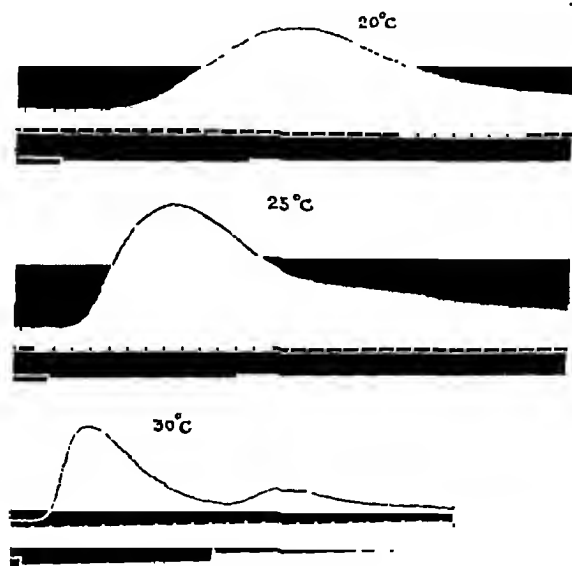


Fig. 3. The effect of temperature on the response of frog muscle to A.C. (10 V./10 sec.); time markings 5 sec.

*Effect of temperature.* Decrease of temperature (experimental range down to 3° C.) decreases the rate of rise of tension and rate of relaxation and increases the latent period of the response to A.C. (Fig. 3) and acetylcholine. Owing to the prolongation of the latent period and diminution of adaptation, the muscle appears to find difficulty in beginning the contraction, and to stop contracting when the stimulus has ceased; the inertia of the contractile mechanism is, as it were, increased. The rate of change of length diminishes with decrease of temperature (Fig. 4).

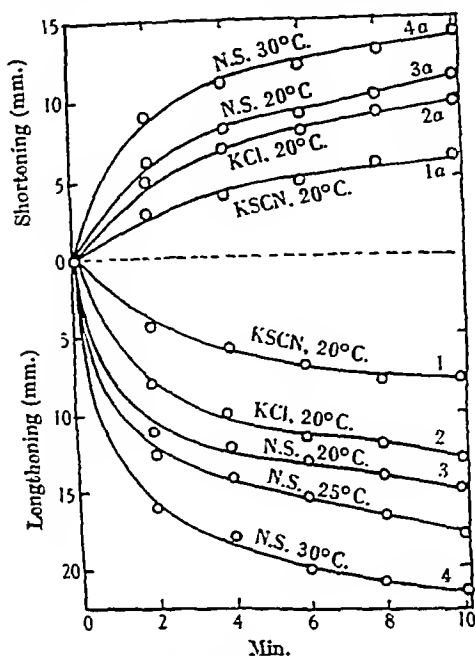


Fig. 4a. The effect of temperature and certain ions on the rate of change of length of frog muscle. N.S. normal saline; KCl, KSCN, replacement of the sodium salt of the saline with these salts.

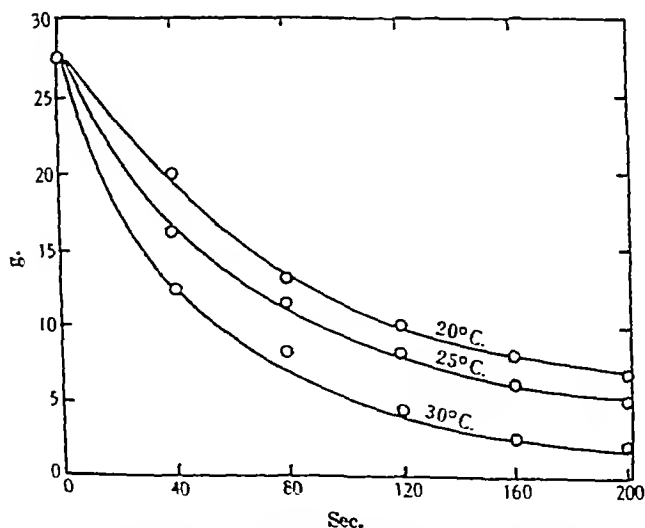


Fig. 4b. Effect of temperature on the relaxation after a.c. stimulation. Measurement of each contraction made from same height from the base line.

With decrease of temperature, the spontaneous contractions and the ammonium withdrawal contractions also become sluggish.

*Effect of ions.* All ions that cause tonic contraction, such as potassium, barium, and the salts of sodium, decrease the rate of rise of tension as well as that of relaxation [Singh, 1938*e*]; the latent period also is prolonged. The effect of these ions is antagonized by increase of osmotic pressure.

Calcium neutralizes the effect of excess potassium. Previous stimulation with A.C. also increases the rate of change of tension during an A.C. contraction; Winton [1937] found that A.C. reduces the viscosity of plain muscle. If the muscle is fatigued by prolonged stimulation, tone develops and the rate of change of tension decreases; Levin & Wyman [1927] found that the viscosity of frog striated muscle was increased during fatigue. If the muscle exhibits much tone, the rate of change of tension is less.

Increase of temperature increases the rate of change of tension, but in excess of ions causing tonic contraction, the rate is diminished at high temperatures (30–45° C.), since the sensitivity to these ions increases; in normal saline, too, the rate diminishes if tone develops at 35–40° C.

Experiments were performed to test the effect of ions on the rate of change of length during isotonic stretch and release, but the presence of spontaneous contractions interfere with the stretch or release curves; these contractions can be diminished by reducing the calcium content of the solutions by 50 %, excluding the potassium chloride and reducing the temperature to 20° C. Under such conditions the spontaneous contractions are usually present, though diminished; but in one muscle they were absent, and in this muscle, potassium and thiocyanate diminished the rate of change of length, though the effect was not very marked (Fig. 4). Barium chloride and low temperatures (3–5° C.) have such a marked effect, that the diminution in the rate of change of length is quite obvious; 0.016 *M* BaCl<sub>2</sub> produces nearly the maximum decrease, which is reversible in 15–30 min., and 0.04 *M* BaCl<sub>2</sub> produces a change, which is only slightly reversible in 2 hr.

In *Mytilus* muscle these ions produced asymmetric curves [Singh, 1938*c*]; in frog muscle asymmetric curves are also produced if sufficient time is not allowed for the process of contraction to subside. The asymmetric curves in *Mytilus* muscle were probably, then, due to the fact that, owing to high sodium chloride content of sea water, adaptation is very slow, and even though considerable time was allowed, the process of contraction had not subsided.

*Effect of strength of stimulus.* The rate of rise of tension increases with the strength of the stimulus. This is comparable to the effect of variation of the load on the rate of extension. Substances that diminish the excitability, diminish the rate of rise of tension, but the rate of relaxation is not appreciably affected. At high temperatures (30–40° C.), the excitability to A.C. diminishes but the rate of rise of tension is rapid; this suggests that the process that produces contraction is undiminished, and that the smaller tension developed at high temperatures is due to some other process, viz. adaptation [Singh, 1938*d*].

*Magnitude of the response.* Winton [1937] found that the viscosity of *Mytilus* muscle during a D.C. contraction does not decrease to the same extent as it does during an A.C. contraction, so he concluded that this accounted for the response to D.C. being smaller than that to A.C. This conclusion finds support from the present experiments, as it has been found that whatever decreases the rate of rise of tension, the rate of relaxation and the rate of change of length, also diminishes the magnitude of the response; at higher temperatures all these factors increase, but the response diminishes owing to increased adaptation, so that viscosity as well as chemical factors are concerned in determining the magnitude of the response.

During tonic contraction the muscle becomes inexcitable to all forms of stimulation; this is probably due to increased viscosity. The responses of the muscle become sluggish and this sluggishness culminates in inexcitability. This is especially seen if *Mytilus* muscle is stimulated with A.C. in saline containing barium. It is also seen if *Mytilus* muscle is stimulated with potassium if previously immersed in *Mytilus* saline the chloride of which has been replaced with bromide or nitrate. Similarly frog muscle becomes inexcitable if it passes into tonic contraction induced by anions.

*Viscosity during contraction.* Winton [1937] found that the viscosity of *Mytilus* muscle was reduced during A.C. contraction.

When the muscle is excited, two processes probably occur: (1) leading to contraction, (2) reducing viscosity. In *Mytilus* muscle, if tonic contraction is produced by barium, these two processes can actually be dissociated [Singh, 1938*f*]. In any case tone, which is probably accompanied by increased viscosity, is reduced during A.C. contraction. It has been suggested previously that calcium reduces the viscosity, and is liberated by the first process. During A.C. contraction, which appears to be produced by ions within the fibres, the viscosity is reduced, and it is significant to note that increase in osmotic pressure, which

increases the concentration of ions within the fibres, also increases the rate of rise of tension and that of relaxation if reduced by excess of potassium.

### *Oxygen consumption*

The oxygen consumption of unstriated muscle has been measured by Meyerhof & Lohmann [1926], Rosenthal & Lasnitski [1928], Lovatt Evans [1926], and Glaister & Kerly [1936]. In the present experiments, the weight of the muscle used for studying the oxygen consumption during tonic contraction varied from 50 to 150 mg.; readings were taken hourly. For A.C. stimulation readings were taken every 15 min., so larger amounts of tissue, consisting of 5-6 pieces of muscle (500-700 mg. of dry weight), had to be used. Use of such large amounts of tissue does not appear to be a drawback as: (1) the oxygen uptake is very low and hence diffusion of gas causes no limitation; (2) the muscles are flat and their thickness was reduced by stretching (with stretching the response to A.C. increases); (3) the muscles were all separated from one another, so that gas exchange was not hindered. The oxygen consumption during the first 2-3 hr. is irregular; besides, the volume of the muscle increases. Thereafter the oxygen consumption becomes steady and remains remarkably constant for several hours; 2½ c.c. of isotonic glucose in 100 c.c. of saline is helpful. The values were recorded during the steady period only.

*Oxygen consumption during normal tone.* Normal tone appears to require oxygen, as shown by the following observations: (1) The effect of stretching: if the length of the muscle is increased, the oxygen consumption of resting and stimulated muscle decreases (Table II, four experiments); this is probably due to the fact that under such conditions tone diminishes. (2) The optimum temperature for tone is 20° C., and if the temperature is decreased from 27 to 20° C., the oxygen consumption of muscles showing high tone, such as in saline the chloride of which has been replaced with nitrate, *increases* (three experiments). (3) The effect of adrenaline; tone as well as oxygen consumption decreases (four experiments).

*Oxygen consumption during tonic contraction.* Two kinds of results were obtained; in one there was increased and in the other decreased uptake of oxygen. During tonic contraction produced by sodium thiocyanate (six experiments), sodium iodide (four experiments), sodium nitrate (three experiments), the chloride of the saline being replaced by the anion in all these experiments, potassium chloride (three experiments), the sodium of the saline being replaced with potassium, and 0.016M

TABLE II. Oxygen consumption of frog stomach muscle (different muscles)

## I. Effect of stretching; 27° C.

	$Q_{O_2} \times 100$	
	Of unstretched muscle	Stretched muscle
(a) In normal saline	16.2	12.4
(b) Cl replaced with iodide	44.4	29.5

## II. Effect of stimulation

Stimulating saline or agent	Temp. ° C.	$Q_{O_2} \times 100$	
		Control muscle in normal saline	Treated muscle
(a) Na replaced with K	27	22.0	29.2
(b) Cl replaced with CNS	27	22.0	35.6
(c) Cl replaced with iodide	27	22.7	33.3
(d) 0.016 M BaCl <sub>2</sub>	27	19.1	23.5
(e) 0.04 M BaCl <sub>2</sub>	27	29.4	20.1
(f) Cl replaced with NO <sub>2</sub>	20	20.7	28.1
	27	27.3	19.3
	37	65.2	79.7
	42	60.0	78.5
(g) A.C. (10 V./10 sec./min. for 12 min.)	27	17.9	34.2
	20	18.6	38.8
	15	5.64	11.2
(h) A.C.	10	3.62	7.24
	25	18.6	37.2
	30	18.0	32.0
	37	30.7	30.7

## III. Effect of inhibition; 27° C.

Saline	$Q_{O_2} \times 100$	
	Control muscle	Treated muscle
(a) Na replaced with NH <sub>4</sub>	23.2	37.0
(b) Isotonic sucrose + 0.01 M KCl	26.7	22.4
(c) 1 in 75,000 adrenaline	24.6	18.1

N.B. With A.C. stimulation, the  $Q_{O_2}$  decreases as the excitability decreases with change of temperature.

BaCl<sub>2</sub> (four experiments), the oxygen consumption increased. During extreme tonic contraction produced by 0.04 M BaCl<sub>2</sub> (six experiments) the oxygen consumption decreased. In 0.016 M BaCl<sub>2</sub>, the muscle may exhibit spontaneous contractions, but 0.04 M BaCl<sub>2</sub> produces tonic contraction, so that the muscle becomes inexcitable and the rate of change of length is diminished to its maximum extent.

Decreased oxygen uptake was also produced by other ions, potassium chloride (one experiment), iodide (one experiment), 0.016 M BaCl<sub>2</sub> (two experiments), potassium thiocyanate (one experiment); these ions are less potent than barium chloride in decreasing the rate of change of length.

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a contracture occurs, and if stimulation with A.C. is applied, relaxation is greatly prolonged (Fig. 5); the rate of change of length is greatly diminished. During such prolonged relaxation, which lasts about 1–2 hr., the oxygen consumption was decreased. In cyanide, the respiration gradually becomes less, but the experiment showed that increased respiration was not necessary for prolonged relaxation.

*Oxygen consumption during inhibition.* Replacement of the sodium of the saline with ammonium produces marked inhibition but the oxygen consumption is increased (three experiments). It has been shown also [Singh, 1939], that the action of sodium chloride in frog muscle is inhibitory and the immersion of the muscle in isotonic sucrose with the usual amount of potassium chloride causes the muscle to pass into contraction, and the oxygen consumption diminishes (three experiments). These experiments show that inhibition is an active process.

Relaxation of the muscle may be (1) passive, caused by subsidence of the contractile process or by decrease of tone, (2) active, caused by a process which is antagonistic to tone, such as by A.C. It appears that a certain kind of shortening of the muscle is associated with decreased oxygen consumption, i.e. that produced by sucrose and barium chloride, and lengthening with increased oxygen uptake, i.e. that produced by ammonium ions.

#### *Effect of certain ions on weight*

In previous papers [Singh, 1938a, b], it was shown that the physiological activities of many solutions varied in the same order as that of their effect in changing the weight and base content of *Mytilus* muscle. As the response of frog muscle to certain solutions is different from that of *Mytilus* muscle, the effect of these solutions on the weight of frog muscle was recorded.

In frog muscle isotonic solutions of sodium chloride (0.120 *M*) cause the muscle to lose weight by 20–30 % in 24 hr.; in *Mytilus* muscle the effect of isotonic sodium chloride (0.564 *M*) is opposite to that in frog muscle. In frog muscle 0.564 *M* NaCl also causes increase in weight, so that, like potassium chloride or calcium chloride, small concentrations of sodium chloride cause dehydration, larger concentrations having the opposite effect. In correlation with this is the fact that small concentrations of sodium chloride, as in frog muscle, are inhibitory, while large concentrations, as in *Mytilus* muscle, are stimulatory.

Calcium chloride (0.0032 *M*) and potassium chloride (0.01 *M*) antagonize the effect of sodium chloride, that is, cause an increase in weight, the effect being opposite to that in *Mytilus* muscle; in correlation with



*Oxygen consumption during A.C. contraction.* In three experiments the muscle was stimulated with A.C., 12 V./10 sec./min. for 12 min.; 3 min. had to be allowed for cooling, since the passage of the current produced a slight rise in temperature. In six observations, the oxygen consumption rose by exactly 100%. The control muscle outside, the dry weight of which was 10% of the experimental muscles, gave an average tension of 30 g. with each contraction.

*Oxygen consumption during relaxation.* Unfortunately, in frog muscle, it is not possible to produce that typical slow relaxation of an A.C. or D.C. contraction which is so easily produced in *Mytilus* muscle; nor has

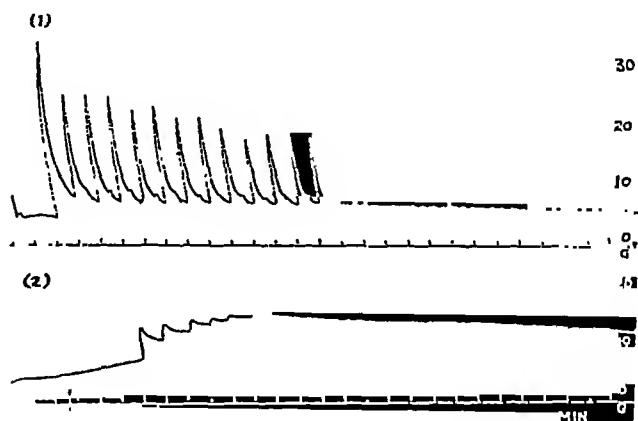


Fig. 5. Upper figure shows frequent stimulation of frog muscle with A.C. 12 V./10 sec./min. for 12 min.; the lower figure shows the contracture produced by cyanide (0.024 M NaCN) and the contractions produced by A.C., followed by slow relaxation.

it been possible to produce a tonic contraction (easily produced in *Mytilus* muscle by barium) consisting of a series of contractions, each being followed by extremely slow relaxation, 10–20 g., taking 60–90 min. to subside. Bayliss [1928] has calculated that with such a contraction no appreciable rise in oxygen consumption would be expected.

The only methods by which prolonged relaxation was produced in frog muscle were: (1) repeated stimulation with A.C. (Fig. 5); the base line rises, and at the end of the period of stimulation gradually falls. Eight observations showed no rise in oxygen consumption during this period; two observations exhibited a rise, but subsequent repeated observations showed that this was due to a general rise in the level of oxygen consumption. (2) If the muscle is treated with 0.024 M NaCN,

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Schultz [1897]. *Arch. Anat. Physiol., Lpz.*; quoted from Winton, F. R. [1927].  
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this is the fact that the effect of calcium in frog muscle is opposite to that in *Mytilus* muscle. When frog muscle is immersed in Ringer solution, it increases in weight for 2-3 hr. and then loses weight; this is due to the calcium of the saline.

The anions, bromide, nitrate, iodide and thiocyanate, cause increase in weight both in *Mytilus* and frog muscles.

### SUMMARY

1. The optimum temperature for tonic contraction in frog muscle is 20° C. and in *Mytilus* muscle 14-15° C.; that for the alternating current contraction in frog muscle is 25-30° C. and in *Mytilus* muscle 20° C. The optimum temperature varies with the composition of the saline.

2. Two factors determine the excitability with change of temperature; one of these is adaptation.

3. Decrease in temperature and tonic contractions diminish the rate of rise of tension during A.C. stimulation, the subsequent rate of relaxation and the rate of change of length on isotonic stretch and release.

4. Normal tone requires oxygen.

5. Most tonic contractions produce an increase in the oxygen uptake.

6. Stimulation with A.C. 12 V./10 sec./min. for 12 min. increases the oxygen uptake by 100 %.

7. Oxygen uptake is not increased during passive relaxation.

8. Oxygen uptake is increased during inhibition produced by sodium and ammonium ions.

9. Isotonic sodium chloride causes frog muscle to lose weight; calcium antagonizes this action.

We wish to express our sincere thanks to Lt.-Col. S. S. Sokhey, I.M.S., Director, Haffkine Institute, for facilities provided.

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The ends of the axon must be tied if the preparation is to be used for any considerable time, as the axoplasm flows out of the ends rather rapidly [Young, 1935], with accompanying loss of irritability. After the ends have been tied off there is a slow progressive loss of irritability away from the ends, without any great alteration of electrical characteristics. This provides a very convenient system for studies on the decrementing of action potentials and their transition into electrotonic waves. The branches which leave the giant axon also provide very convenient partial blocks for experiments on decremental and incremental conduction.

#### METHOD OF PREPARATION

The material used was fresh *Loligo pealii*, obtained at the Marine Biological Laboratory, Wood's Hole, Mass., in August 1937. The nerves were usually dissected soon after the squid had been caught, although in some cases the animals were kept in the aquarium for one or two days before being used. The whole nerve was first dissected under a low-power microscope with transillumination through the muscles. Having been tied at the ends, it was removed from the body and soaked in sea water for a few minutes. If the giant axon was to be isolated the nerve was then fastened by its end ties to two pins and the small fibres, and as much as possible of the connective tissue around the axon, removed under sea water with iridectomy scissors and micro-forceps. The teased fibre was then further washed in sea water before mounting. No apparent harm was done even by several hours of soaking.

The moist chamber used was that illustrated in Fig. 1. It consisted of a brass plate upon which four brass strips were mounted edgewise so as to form a shallow box about 10 cm. wide, 12 cm. long and 2 cm. deep. The box was sealed by a glass cover stuck on with petroleum jelly, a glass plate being waxed into the bottom of the brass box to make it watertight and to prevent the nerve coming in contact with the brass. A small slot was milled in the bottom of the brass chamber to permit the nerve to be illuminated from below, and studs were fitted to the bottom of the chamber so that it might be attached to the stage of the microscope for close observations of the adjustment of the electrodes, and for measurements of birefringence. Large blotting-paper pads soaked with water were attached to the glass bottom plate to keep the interior of the chamber moist.

Through opposite sides of the chamber four electrode rods were installed on sliding ball and socket joints in such a manner that there was freedom of movement of the electrodes, back and forward, about the

## PARTIAL EXCITATION AND VARIABLE CONDUCTION IN THE SQUID GIANT AXON

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(Received 1 August 1939)

FOR study of the detailed mechanism of the nerve impulse it is necessary to be able to investigate the behaviour of single axons. It is possible to obtain records of the electrical responses and of the excitation characteristics of single nerve fibres in small nerve bundles by the technique of Blair & Erlanger [1933] or in teased single fibre preparations by the technique of Kato [1934] for medullated fibres and of Hodgkin [1938] and Ledingham & Scott [1938] for crustacean fibres. Yet these methods of isolation from a nerve trunk are always laborious and liable to alter the physiological state of the fibre. Certainly the techniques do not lend themselves easily to the correlation of excitation results with measurement of gross characteristics such as electric impedance, respiration, birefringence, and X-ray structure.

The difficulty can be overcome by the use of the very large axons in the stellar nerves of the squid (*Loligo*) [Young, 1938; Pumphrey & Young, 1938]. Here the giant axon constitutes over a quarter of the total volume of the nerve, being usually about 0.5 mm. in diameter in the longest stellar nerve of *L. pealii*.

The threshold of the giant axon for electrical stimulation is so much lower than that of the other fibres and its response so dominates the total electrical response of the nerve that for many purposes it is unnecessary even to tease the giant fibre free of the surrounding smaller fibres; thus injuries due to manipulation are reduced to a minimum. With a little practice, however, it is possible to obtain preparations of the giant fibre teased free of smaller fibres and of much of its connective tissue investment and capable of conducting impulses for hours after dissection. Any observations made on the giant fibre after isolation can therefore be controlled by studying also its response *in situ* in the nerve.

electrode was not essential, while No. 36 B. and S. wires (0.127 mm.) were used when accurate position measurements were being made.

The nerve or giant fibre was placed upon the electrodes so that it sagged slightly but definitely; in this position there was neither great tension on the fibre nor was it bent too acutely over the electrodes. Too sharp kinking was found to be surprisingly effective in damaging the nerve locally. The ends of the nerve were usually supported by attaching the threads tying the ends of the nerve to small insulated hooks in the ends of the chamber. In experiments involving immersion of the nerve in saline, the nerve was simply lowered on to the floor of the chamber and a puddle of suitable dimensions built up on the waxed glass floor.

The nerve chamber was unthermostated so that the temperature varied through fairly wide limits between various experiments, but in any one experiment was fairly constant as the temperature of the closed room changed only slowly. As temperatures tended to be high (approaching 38° C. in some cases) this factor must be considered before comparing these results with those obtained in the cooler climate of Plymouth, England. Later experiments, indicate, however, that the temperatures reached were not sufficiently high to alter the results seriously.

#### ELECTRICAL TECHNIQUE

Since all the experiments to be undertaken in this investigation required direct synchronization of the stimulus with the cathode-ray sweep, a modified version of the all-electric synchronizer [Schmitt, 1934] was used in conjunction with an improved thyatron stimulator and a cathode phase inversion high-gain amplifier, the whole being built into a compact relay rack unit to be easily portable.

The synchronizer design permitted stimulation at any frequency from 1 per min. to 1000 per sec., and also provided means for stimulation irregularly using a key or commutator. In all cases it permitted adjustment of sweep velocity completely independent of sweep frequency and also caused the stimulus to occur at a chosen point on the screen independent of sweep frequency and velocity. As this synchronizer unit also supplied energy to the cathode-ray unit, to the high level amplifier and to the stimulator, its operating potentials were gas discharge regulated to give high stability.

The thyatron stimulator was of a new design which maintained the strength of shock constant independent of frequency of stimulation up to 1000 per sec., although permitting adjustment of shock strength through a ratio of 100:1 by means of a logarithmic *T* pad attenuator. A fine

axis of the rod, and free rotation about the ball joint. The electrode rods were of brass, but were insulated from the actual electrode wires by short bits of ebonite at their tips. This arrangement made it possible to move the electrodes even when high amplification was being used, as the electrode rods served only as mechanical members and were electrically

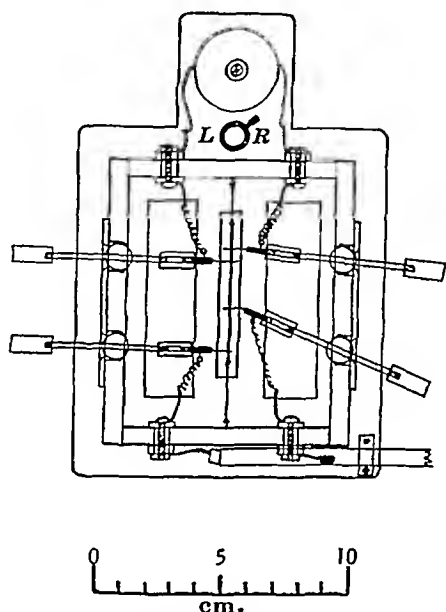


Fig. 1. Moist chamber. The moist chamber is made up of brass strips mounted edgewise on a brass bedplate, and has a glass floor and a removable glass cover. Four movable electrodes are fitted, the two upper ones for stimulation, the lower two for leading off; each is mounted through a sliding sleeve, and ball and socket joint to allow of free movement. Electrical insulation is internal to permit perfect shielding, thus making it possible to manipulate the electrodes with high amplification. Connexion with the electrodes is maintained through highly flexible phosphor bronze springs. The stimulating transformer and its reversing switch are mounted directly on the chamber to reduce capacitive troubles, and the whole chamber can be mounted directly on the dissecting microscope, illumination being provided through a slot milled in the brass bedplate. Humidity is maintained by blotters soaked in distilled water and stuck on the glass floor.

earthed. Contact with the electrode wires was established through slender phosphor-bronze extension springs which communicated with terminals mounted in insulating bushings through the ends of the vessel.

The electrodes themselves consisted of short pieces either of platinum or of chlorided silver wire fixed into the ebonite insulators. No. 22 B. and S. gauge (0.64 mm.) wires were used whenever sharp location of an

The whole unit of photocell and amplifier was then inserted into the draw tube of the microscope previously focussed on the nerve. The sensitivity of this unit was such that it would readily "see" a  $\frac{1}{2}$  W. neon tube at a distance of thirty feet without any light gathering lens system.

### RESULTS

The ordinary propagated action potential in the squid axon shown in Fig. 2A has constants strikingly similar to those obtained from frog sciatic A fibres. The wave lasts about 1 msec., rises to its peak within about  $250\mu\text{sec.}$ , and travels with a velocity around 20 m./sec. in a  $500\mu$  fibre at  $20^\circ\text{C.}$  The potential for the giant fibre when teased free averages about 45 mV. although it may increase to 65 mV. or more upon judicious drying of the fibre. In the unteased nerve, this potential is reduced by less than half, thus indicating the relatively large contribution of the giant axon to the total response.

The response of the small fibres is of quite different order of magnitude from that of the giant fibre in speed of conduction, value of potential, and threshold (Fig. 2B), their conduction rate being of the order of 4 m./sec. and their threshold some 5-10 times higher than that of the giant fibre for a stimulus of minimal energy for the latter. There is therefore little chance of confusing the two responses and consequently the unteased nerves can be used in most experiments.

The giant axon is capable of responding regularly to frequencies of stimulation up to about 480 per sec. at  $25^\circ\text{C.}$  although at this frequency the response diminishes within a few seconds and finally is no longer propagated. At 540 responses/sec. (Fig. 2C) the nerve responds a few hundred times with near normal potential and then gives a rapidly decreasing propagated response which passes discontinuously into the mere electrotonic response. In the figure the first heavy line indicates the initial response during which the camera shutter was opened but briefly and then reopened as the response began to fail. This failure is almost certainly due to progressively decreasing safety ratio in the nerve.

The probability of a variable local response makes for a difficulty in terminology, since it makes ambiguous the term safety factor, ordinarily rather loosely defined as the excess fraction of exciting potential, present at a point on the membrane, to that which will just excite. With the possibility of a local partial response it becomes probable that each point on the nerve membrane may yield a response which varies not only with the current or voltage exciting it, but also with the time sequence of the applied stimulus. Consequently, the term safety ratio has been used throughout this paper. It is defined as the ratio of the output of a unit area of membrane or a unit length of fibre to the lowest input excitation which would, under the same conditions, produce an output equal to itself, these quantities being expressed in terms of whatever variable is felt to be pertinent; thus, for example,



adjustment was also provided which furnished a constant proportional increment of 10 % at any setting of the main dial to permit accurate setting of shock strength. Usually shocks with a stimulating phase of  $40\mu\text{sec.}$  duration were employed, although they could be adjusted at will from about 20 to  $200\mu\text{sec.}$

Since most of the work was done with brief thyatron induction shocks, a shielded air-core transformer with very low capacitance to earth and between windings was mounted on the brass bedplate of the nerve chamber and was connected to the appropriate terminals by thin bare wires, thus rendering the artefacts due to capacitative leakage from the stimulating system practically nil for low-resistance squid nerve. A reversing switch was also mounted on the brass bedplate so that the transformer primary could be reversed without making asymmetric alterations of the connexions to the shielded cable supplying the stimulating transformer.

The R.-C. coupled amplifier consisted of two moderate-gain pentode stages yielding 90-fold amplification each, followed by a low-gain (33-fold) high-level amplifier feeding the cathode-ray tube. If desired, an extra triode stage with a gain of 30 could be added between the second and the final stage. The first two stages were battery fed, while the last stages derived their power from the synchronizer filter. As cathode phase-inversion [Schmitt, 1938] was used in the last stage, there was little distortion even with large signals, so that the cathode-ray deflexions could be used as a measure of input potential without correction. A push-pull circuit was not used throughout, as this would have increased both the cost and the battery current consumption of the unit prohibitively. The frequency characteristic of the amplifier was designed to be linear from 2 up to about 7500 c./sec., where it was arranged to cut off sharply to avoid useless background noise at high amplification. Efforts were also made to prevent distortion due to partial blocking [Schmitt, 1937] by large pulses so that the tails of potentials could be examined in detail even after rather large spikes.

The leads to the amplifier were usually taken through short lengths of ordinary antcapacitance shielded cable, although, in cases where this slight capacitative load was considered undesirable, a very low capacitance head amplifier was brought into play and the nerve was laid directly on the silver chlorided tip of the acorn, head-amplifier valve which served primarily as an impedance changer rather than as an amplifier.

For the experiments on birefringence, a sensitive photocell either of the photo-voltaic or of the gas type was fitted on to this head amplifier.

The whole unit of photocell and amplifier was then inserted into the draw tube of the microscope previously focussed on the nerve. The sensitivity of this unit was such that it would readily "see" a  $\frac{1}{2}$  W. neon tube at a distance of thirty feet without any light gathering lens system.

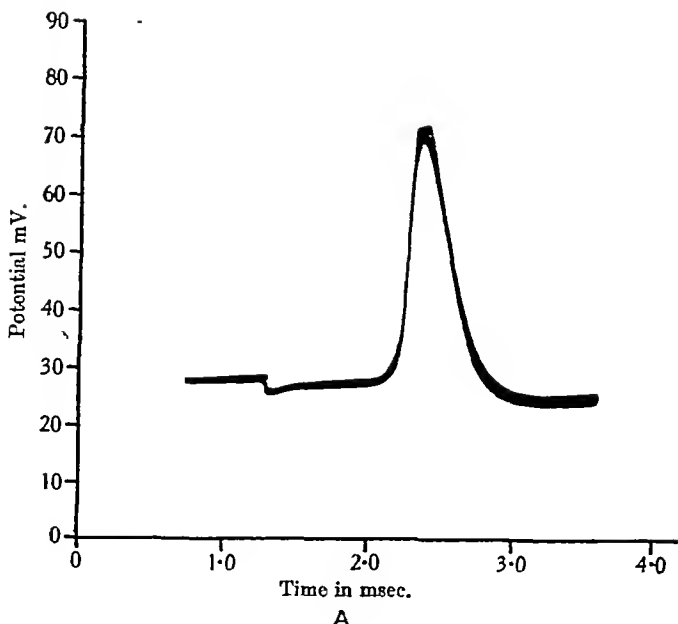
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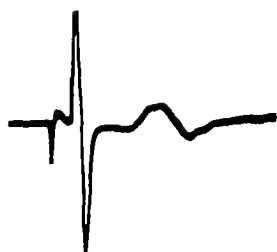
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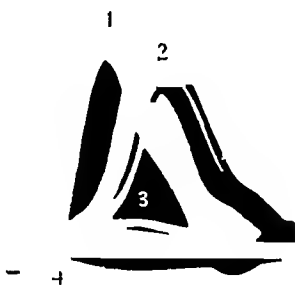
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A



B



C

Fig. 2

- A. Typical monophasic action potential. The action potential in this case travels at a velocity of 19.3 m./sec. at about 22° C. in a 490  $\mu$  fibre and has characteristic constants very similar to those of large frog sciatic fibres.
- B. Separation of giant and small fibre responses. The small fibres conduct so slowly and contribute so little to the total action potential that they are very readily distinguished. With conduction distances of a few centimetres they become entirely separate in time. The threshold of the small fibres is so high that ordinary strengths of stimulation for the giant fibre do not excite the small fibres.
- C. High frequency response. The nerve is stimulated with adequate shocks at a frequency of 540 per sec. and the response led off from a point a few millimetres from the stimulation. For the first few stimuli there is full normal response (1), then for the next four stimuli there is a conducted response at progressively reduced potential and velocity (2). There then follows a local response which is propagated decrementally (3) and finally the electrotonus travelling faster than even the first conducted action potential.

current, voltage, quantity of electric charge, energy, or even quantity of chemical activator can be used depending upon which of the numerous ideas of nerve conduction is held. A large value of safety ratio leads to fast, positive conduction, while a reduction to less than unity means that the tissue is practically inexcitable.

A second unit, the propagation ratio, is also frequently useful. It is defined simply as the ratio of output to input of an element of nerve fibre in any specified set of conditions. This quantity is unity for steady conduction, fractional for decremental conduction, and greater than unity for incremental conduction.

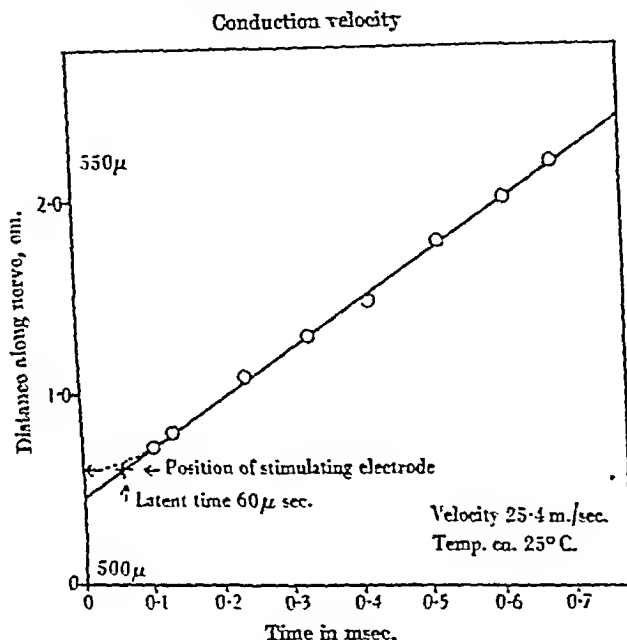


Fig. 3. The nerve is stimulated at a fixed point (0.8 cm.) and the time at which the impulse reaches various points along the nerve plotted. Despite some 10 % taper in the nerve and despite passing a cut branch, there is little variation in the velocity.

It might be supposed that the giving off of branches and the tapering of the giant axon might give rise to considerable variations in conduction velocity but in the uninjured nerve there is striking constancy (Fig. 3), the velocity remaining constant within a very few percent along several centimetres despite a 10 % taper in diameter and a fairly large branch in the middle of the region. The measurements for the figure were made from a series of separate photographs taken as the electrodes were moved with the shock held accurately constant. This is not interpreted as indicating that conduction velocity is necessarily independent of diameter but rather to prove the reasonable uniformity of conduction despite cut branches and irregular wetting.

## DECREMENTAL POTENTIALS

At the points where branches of the giant axon have been severed, the fibre becomes inexcitable more rapidly than in the intervening stretches,

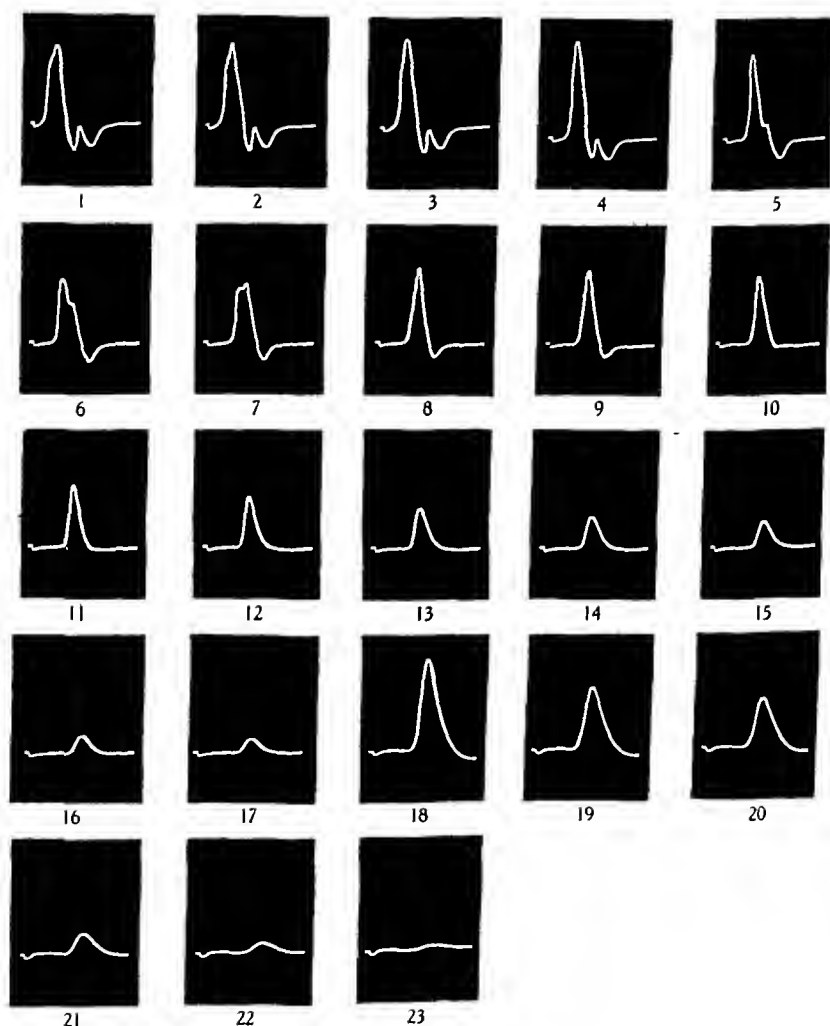


Fig. 4. Blocking of impulse at cut branch. 1-7. Lead ahead of cut branch. 8-11. Decremental conduction. 12-23. Electrotonus. Amplification increased at No. 18.

and consequently if one chooses the proper time after dissection one can use the cut branch point as a convenient partial or total block without

the application of any external agency such as cold, drying, or polarizing current [Hodgkin, 1937].

The variations which the action potential undergoes in the vicinity of such a cut branch which has just sufficiently damaged the nerve to block conduction is illustrated in Fig. 4. The nerve is adequately stimulated far ahead of the cut branch and the series is taken as the recording electrode is moved from ahead of the branch to a point sufficiently beyond it for even the electrotonus to be very small. It will be seen that until the recording electrode reaches the damaged spot, the only distortion of the action potential is a kink which progresses from the tail up toward the crest of the wave. When the kink reaches the first point of inflection of the action potential, however, full propagation stops and there remains only a short region of decremental conduction followed by exponential decay typical of pure electrotonus.

In Fig. 5 is shown a multiple exposure of the potential immediately beyond a partial block just as the nerve is becoming sufficiently damaged to prevent the passage of the propagated impulse. It will be noted that in the first response there is a rather large initial stimulating phase getting through the block and that consequently the impulse goes on propagating with small delay. As the point becomes more nearly blocked, there is a very considerable delay before propagation occurs and finally there is no propagated impulse, but only the small electrotonic spread past the block.

If the nerve, in the condition where it can no longer propagate through the block of itself, is helped by a cathodal shock in the vicinity of the block, it is found that the impulse may still be "pushed" through the block by a stimulating current far too small to stimulate through the block directly. Fig. 6*a* represents the electrotonic impulse recorded beyond a block when the normal impulse is flung up against the block, and 6*b* the response when it is helped through the block by a small locally added impulse.

In the vicinity of a severed branch block which is just sufficient to prevent the passage of the impulse it is quite easy to demonstrate the gradual rather than sudden transition which takes place from propagation to electrotonic spread. In Fig. 7, plotting the logarithms of the potential against the distance along the nerve, it is seen that the action potential comes practically undiminished to the point of injury (this point is only approximately marked) where it begins to decrease, but not until nearly 5 mm. beyond the block does it fall linearly on the logarithmic graph as truly electrotonic potentials usually do. This

phenomenon is most readily explained on the basis of partial excitation; there being a region where the impulse is partly sustained by local physiological activity.

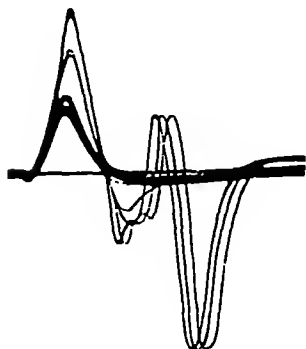


Fig. 5.

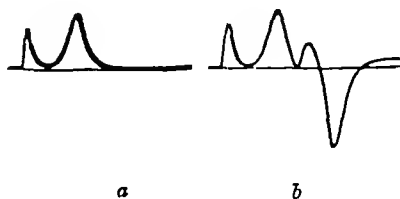


Fig. 6.

Fig. 5. Impulse pushing through partial block. Multiple records are made of the potential just beyond the partial block produced by a cut branch as the block becomes complete. With a light block a large exciting phase is seen and consequently the propagating phase soon re-establishes itself. With increased block the exciting phase becomes smaller and earlier but is still followed by propagation at later and later times. Soon there is only decremental conduction beyond the block and eventually only electrotonus. This process correlates closely with that illustrated in Fig. 2C for artificially reduced safety ratio.

Fig. 6. Stimulus assisting impulse through block. *a*. With a local injury just sufficient to block the propagated impulse started by a threshold stimulus the record *a* is obtained. The first peak represents shock escape and the second the exciting phase from the impulse. *b*. If the stimulus is now increased, its effect, despite its application several millimetres before the block, sums with the local action potential to re-establish propagation beyond the block resulting in the additional diphasic response in *b*. The delay in re-establishing propagation is very apparent.

Perhaps a clearer, although less quantitative, demonstration of this phenomenon is seen in Fig. 8, where the normal progressive loss of excitability inward from each severed end of the nerve is used instead of a cut branch to give decremental conduction. The characteristic slow fall of potential preceding fully electrotonic conduction is seen in each case although, in the time which has elapsed between the two sets of records, the loss of irritability has progressed some 10 mm. up the nerve.

As a control on the shape of the previous two curves, subthreshold stimuli were applied to a normal nerve, and the electrotonus read off at

various distances from the stimulating electrodes, with the result shown in Fig. 9. The potential fell away almost exactly exponentially from the

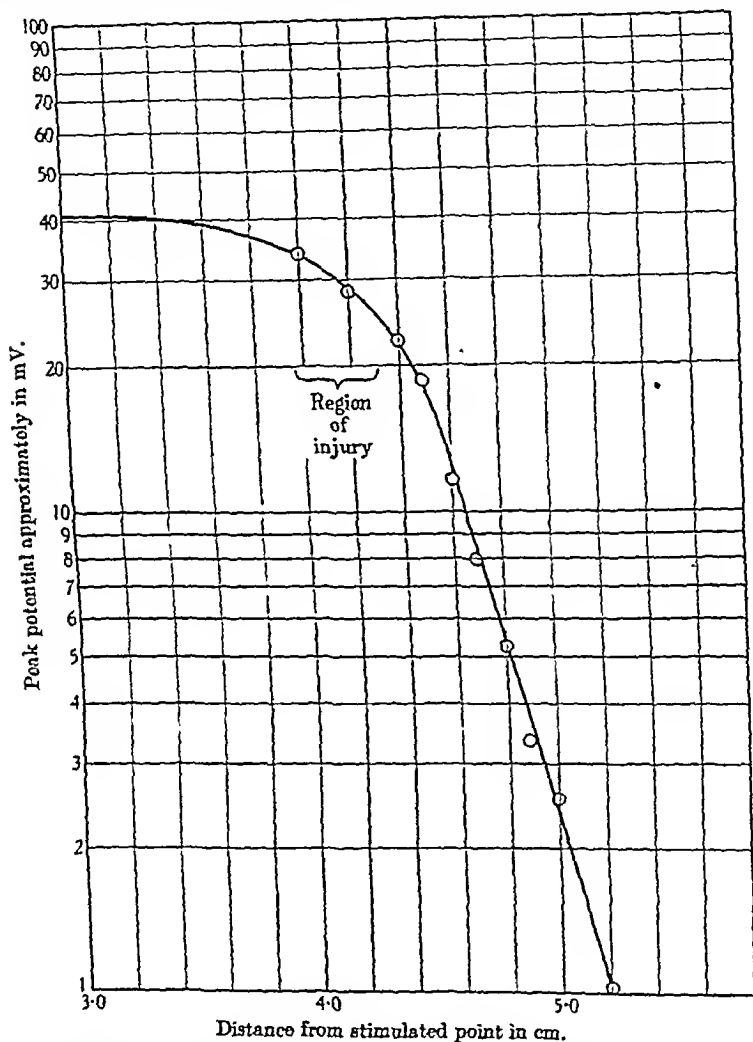


Fig. 7. [Decremental conduction and electrotonus. The impulse travels down the uninjured portion of the nerve with constant peak potential. Upon reaching the injured region it decrements slowly as some local activity is present but then falls more rapidly along the log curve as only electrotonus persists. Distance for attenuation to  $1/2$ , 1.88 mm. to  $1/e$ , 2.5 mm. Fibre diameter  $490 \mu$ .

start; there was in fact some tendency to fall more rapidly than exponentially at first.



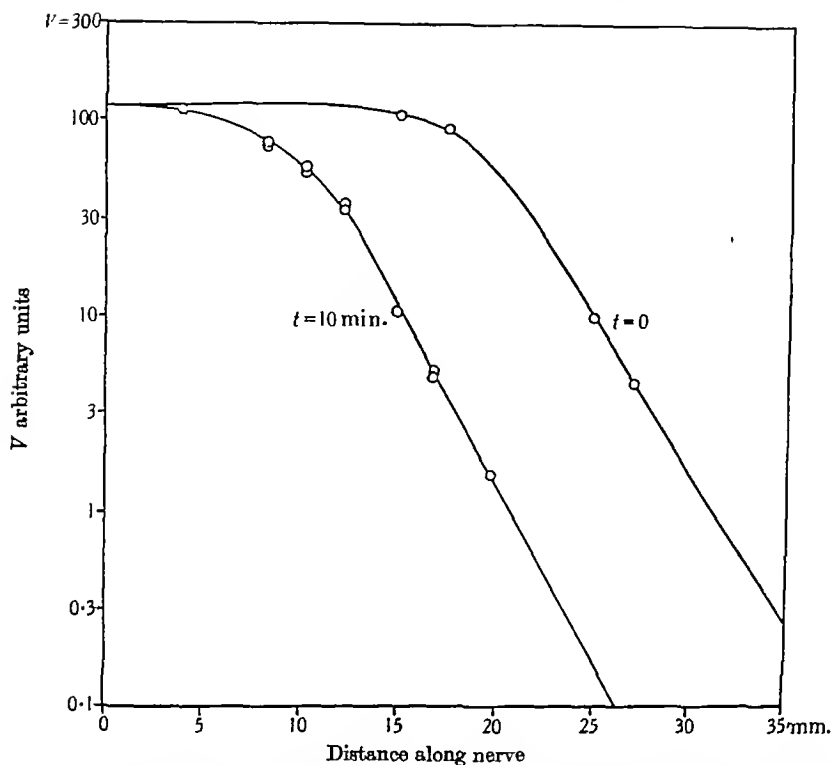


Fig. 8. As the nerve dies progressively from the ends inward, the regions of decremental propagation and of exponential decay move nearer the centre of the nerve. The shape of the curve relating log of peak potential to distance along the nerve and the exponential decay constant, however, remain constant.

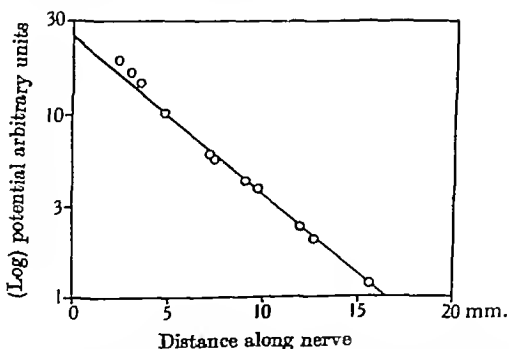


Fig. 9. Decay of electrotonus from subthreshold stimulation. A fresh nerve was stimulated with stimuli too small to evoke even local responses (about 0.3 threshold) and the electrotonus measured at various distances. The fall is exponential from the first.

## LOCAL EXCITATION

If stimuli of near threshold strength are applied to a squid giant fibre and the potential at various distances from the stimulating electrodes recorded, it will be found that as the distance from stimulator to pickup lead is decreased, the threshold becomes progressively less clearly defined. At relatively large distances, of the order of a centimetre or more,

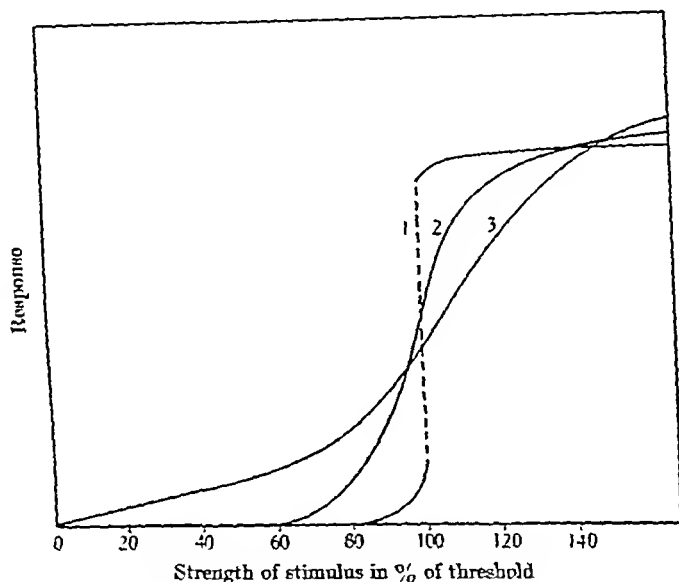


Fig. 10. Development of local potential. Curves 1, 2, and 3 represent diagrammatically the variation of peak potential with strength of stimulus in terms of threshold taken at positions progressively closer to the stimulating electrode. Close to the source of stimulus (3), pure physical response passes over into physiological response smoothly and continuously. At great distances the response becomes practically all or nothing (1) with a definite threshold and a discontinuous rise.

there is no appreciable gradation of response, the response arising instantaneously from a very small "electrotonic" potential to full action potential at a sharp threshold and then rising only minutely with stronger stimuli. On approaching within a few millimetres of the stimulus the gradation is more evident, there being a well-defined linear portion where the response is proportional to the stimulus, the purely electrotonic region; then a disproportionate rise leading up to a discontinuity after which a slight but definite increase can again be obtained with stronger stimulation (curve (3), Fig. 10).

At a certain critical distance, depending upon the length constant of the nerve and the safety ratio, there comes a time when the potential no longer increases discontinuously (curve 2). Still closer to the stimulus the initial rise of polarization is difficult to follow due to the reflected

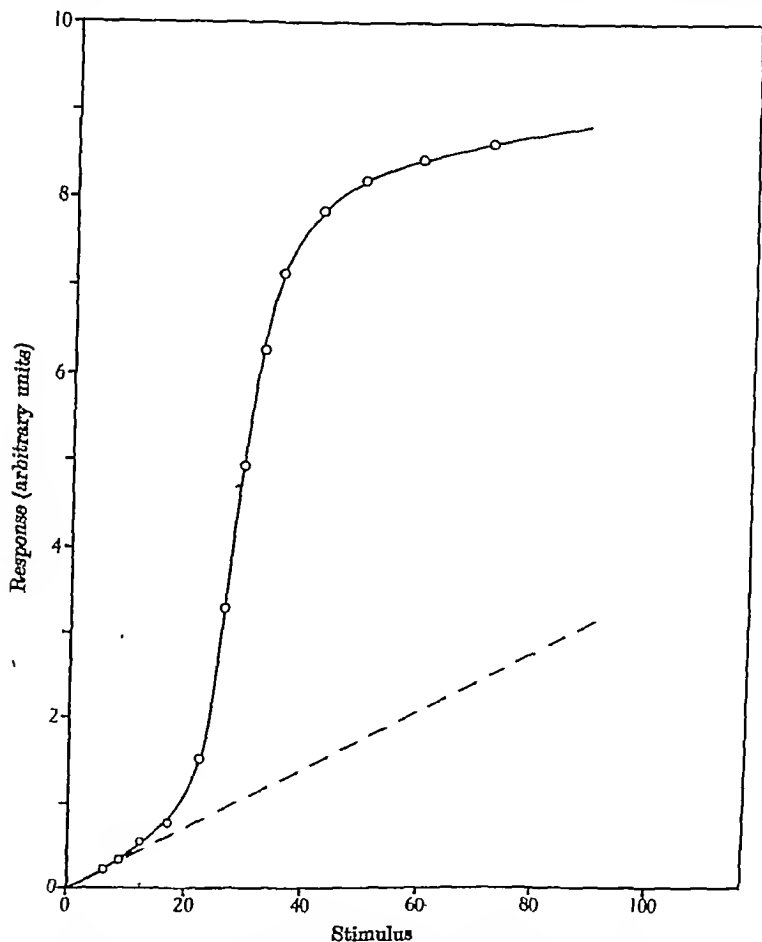


Fig. 11. Peak value of local response potential very close to stimulating cathode for a nerve just unable to conduct.

phase of the full-grown action potential from remote parts of the nerve but if the safety ratio of the nerve be reduced by drying or by aging, this local phenomenon may be studied at leisure since it is now impossible to start a propagated impulse, hence there can be no reflected wave. By this technique curves of the form shown in curve 3 are inferred for points

close to the stimulated region. There is first a linear rise of polarization proportional to the stimulus and consequently probably only passive, then an abrupt rise which is certainly the physiological local response, and finally a slow steady rise, nearly proportional again to the increase in stimulus, and probably due to the purely physical effects summed with a slight increase in local response due to the excitation of slightly more remote regions of nerve. Fig. 11 illustrates quantitatively this stimulus-response curve very close to the stimulating electrode in the case of a nerve just unable to respond.

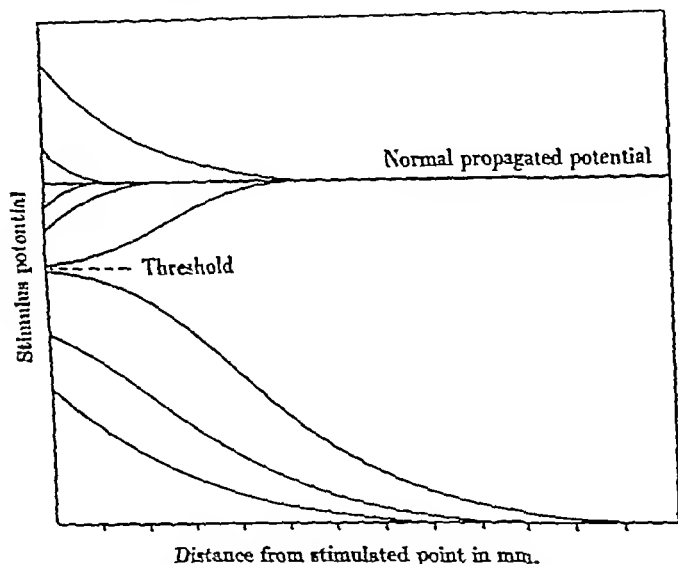


Fig. 12. Spatial distribution of potential. For stimuli of less than threshold strength, the potential decrements rapidly although showing a tendency to decremental propagation when very near threshold. When even very slightly superthreshold stimuli are applied, the potential quickly builds up to the normal propagating value. Very strong shocks increase the potential locally but it then decays with about the same space constant as do very weak shocks to the normal propagating level. As the curves are only qualitative, the distances marked indicate order of magnitude only.

The spatial distribution of peak potential is illustrated diagrammatically in Fig. 12 where peak potentials are plotted as a function of distance from the stimulus with strength of stimulus as a parameter.

Actual photographic records of the sort of potentials which are measured for the construction of Fig. 12 are shown in Fig. 13 which is arranged to resemble Fig. 12, the four rows representing four typical strengths of stimulation;  $\frac{1}{2}$  threshold, just subthreshold, just threshold,

and super-threshold; the columns representing distances from the stimulus of 1.8, 2.5, 4.8, 7.6, and 11.6 mm. respectively.

A series illustrating the development of local potential in a good nerve near to the stimulating electrodes is given in Fig. 14. Here the rise of local potential and the re-entry from remote regions as decrementless propagation sets in are clearly seen. The strengths are in terms of percent threshold. It is interesting to note that in nerves with reduced



Fig. 13. Spatial distribution of local potential. The records in rows *a*, *b*, *c*, and *d* represent the potentials resulting from shocks of super threshold, just threshold, just subthreshold, and half threshold respectively. The figures in columns 1, 2, 3, 4, and 5 represent potentials recorded at distances of 1.8, 2.5, 4.8, 7.6, and 11.6 mm. respectively.

safety ratio, there may be a region of a centimetre or more in which incremental or decremental conduction in all gradations may be produced simply by gradation of stimulation intensity. In such nerves the local physiological response beneath the stimulating electrode may be well into its falling phase before the re-entrant impulse appears.

Because of the large dimensions of the squid nerve, it is possible to stimulate with transverse currents as a large potential drop can exist between the two sides of an axon without very large currents flowing. As this sort of stimulation tends to stimulate only a limited area of the nerve membrane, it probably sets up excitation in an irregular pattern

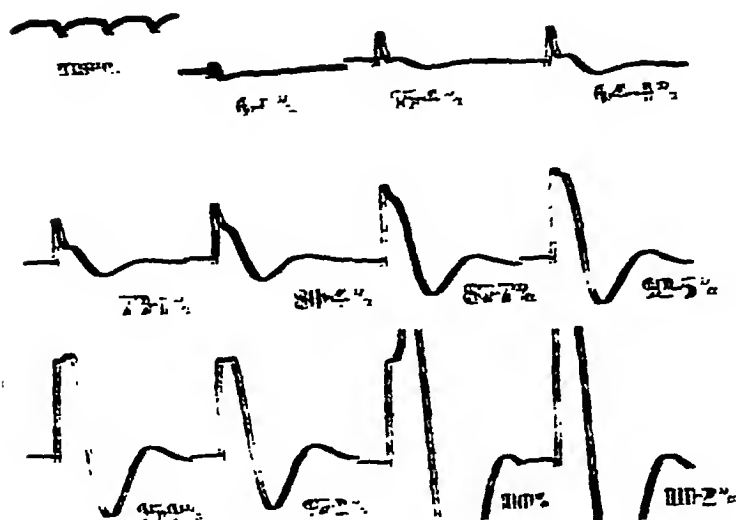


Fig. 14. Development of local potential with increasing shock strength. The strength of stimulation is given in percent of threshold. The time equal 1  $\mu$  sec. is the potential being recorded 0.25 mm. from the site of stimulation.

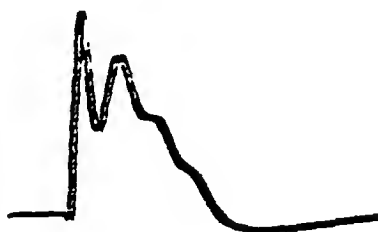


Fig. 15. Response of nerve to transverse stimulation.

along the side of the nerve which finally serves to set off a propagated impulse. A record of the response of a fibre of living frog skin, which was subjected to the sort of stimulation with decreasing lead 1 shock distance 0.1 mm. only is illustrated in Fig. 15.

EFFECT OF ACTIVITY ON THE BIREFRINGENCE OF THE AXON<sup>1</sup>

The axon of a fresh squid giant fibre shows weak birefringence which, like most structures composed of oriented protein fibrils, is positive with respect to the long axis of the fibre. The axon birefringence has been shown to be due largely to form birefringence arising from the presence of a very low concentration of oriented submicroscopic protein rodlets [Bear, Schmitt & Young, 1937].

To test the possibility that the propagation of the action potential involves a transitory change in orientation of axoplasm constituents, a sensitive photoelectric method was devised which is capable of recording extremely small changes in birefringence, such as would result from changes in orientation of the axoplasm proteins, without appreciable time lag. With an intense light source and an ordinary ocular in the draw tube of the polarizing microscope the giant fibre was brought into focus between crossed Nicol prisms, the long axis of the fibre being oriented at  $45^\circ$  to the planes of polarization of the Nicol prisms. With suitable objective the axon could be made to cover the entire field, thus increasing the sensitivity. Experiments were also done after partial compensation of the fibre birefringence with a suitable compensator. The ocular was then removed and the photocell with amplifier inserted into the draw tube of the microscope. Calibration of the system showed that with full usable amplification a change of 0.00025 % of the light intensity incident on the photocell could be detected on the oscillograph provided the change occurred slowly. If the change occurred in 0.1 msec. the sensitivity was halved. Assuming that the fibre thickness does not change appreciably during passage of the impulse these figures express also the minimal change in retardation and of birefringence which could have been detected. Since the cathode-ray sweep was synchronized with the stimulus, any change in birefringence lasting more than  $20\mu\text{sec.}$  would be recorded as a standing pattern on the cathode ray tube as the nerve was stimulated repetitively.

Although the experiment was tried under a variety of conditions, no change in birefringence could be discovered either in the initial, rapid phases of the action potential or in the late recovery phases. It is of course still possible that changes in birefringence do accompany the active process but the present experiments set an upper limit to the magnitude of the process if it does occur. For the rapid phase the change cannot be less than 2 % of the initial birefringence, and for slow

recovery processes they cannot be more than 0.08 %. Since, as was pointed out [Bear *et al.* 1937], the birefringence of the axon is due to only a very small fraction of axon constituents, and since very small changes in this birefringence could have been detected in the present experiments, it is unlikely that impulse propagation involves any appreciable change in the configuration of the oriented components of the axon.

### DISCUSSION

From the continuous way in which the "propagation" phenomena in squid fibre merge with the properties commonly termed "electrotonic", there seems little likelihood that these classifications are fundamentally distinguishable. Usually the distinction between these two categories is given as the presence or absence of "local physiological activity" at the nerve membrane. This criterion is perfectly satisfactory in the cases of very subthreshold stimulation or of definitely adequate stimulation, but there is undoubtedly a middle zone where propagation may start electrotonically or propagation degenerate into electrotonus.

So far there is no crucial experiment to decide definitely whether an element of nerve membrane can respond partially or whether it must respond all or none, yet it is certain that a nerve fibre, as a whole, can exhibit partial responses.

One experiment which is rather convincingly in favour of local partial excitation is that in which a fibre, in a uniformly depressed state, is stimulated with near threshold stimuli and the response is found to increment or decrement only very slowly over a considerable length of nerve and can be made to conduct at a wide variety of potential levels. It may be argued that there is a mosaic structure with varying thresholds at various points, thus resulting in a distribution of local thresholds. If the mosaic structure is fine, however, as it is likely to be, this distribution of thresholds would be indistinguishable from a smoothly variable response and may properly be treated as such. In any event, more specific data must be obtained relating directly to the problem before a valid conclusion can be drawn.

### SUMMARY

1. The action potentials from giant axons of the squid, both teased and in their nerve trunk are examined and shown to be very suitable for single fibre experiments, conduction velocities being about 20 m./sec. at 22° C. and the potential from the single giant axon, 40 mV. or more.
2. The nerve preparation (by frequent stimulation or by drying) can be placed in a state of reversible reduced safety ratio to permit easy



observation of local potential. Convenient partial blocks are provided at places where branches are cut off and the fibre also dies gradually and progressively from the ends inward, thus providing a natural killed lead and a place for measuring purely electrotonic effects.

3. Partial excitation is demonstrated in three ways: (a) by the decremental rather than electrotonic decrease of potential beyond a partial block just sufficient to stop propagation, (b) by incremental and decremental conduction in the vicinity of an electrode providing near threshold shocks, (c) by non-linear responses from a nerve much too depressed to conduct.

4. Temporal and spatial distributions of potential with intensity of shock as a parameter are illustrated.

5. Transverse stimulation of the squid nerve is demonstrated.

6. No change in birefringence was detected in the axis cylinder of squid giant fibres either in the initial fast phase of the potential response or in the slow recovery phases, although the sensitivity of the method was such as to be capable of revealing changes of the order of 0.1 % of the birefringence of the resting axon.

7. The evidence suggests that the local response, even of small elements of nerve, is graded rather than all or none.

8. Two units of local activity termed the "safety ratio" and "propagation ratio" are defined. These are useful for quantitative description of the activity of a nerve element irrespective of the theory of conduction held.

We should like to thank Dr J. Z. Young and Dr Bernhard Katz for reading the MS.

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# CORRELATION OF LOCAL EXCITABILITY WITH LOCAL PHYSIOLOGICAL RESPONSE IN THE GIANT AXON OF THE SQUID (*LOLIGO*)

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THE passage of an electric current through part of a nerve produces local changes in the state of the fibres even though the current is too small to set up a propagated nerve impulse. Of these changes the most interesting for the elucidation of the mechanism of the nerve impulse are (1) the changes in excitability [see especially Katz, 1937; Erlanger & Gasser, 1937] and (2) the changes in electrical potential which occur in the fibres close to the electrode through which the conditioning current is applied [Hodgkin, 1937, 1938; Ledingham & Scott, 1938; Schmitt & Schmitt, 1940].

It has been suggested that the variations in excitability can be correlated with local electrical potential changes in the fibres. But the changes in excitability and the changes in potential have never been studied side by side in the same preparation. Indeed the only previous observations of the effect of a subthreshold shock on the local potential produced by a subsequent shock are those of Hodgkin [1938] on the local potentials produced during repetitive stimulation. Excitability changes have been followed chiefly in bundles of medullated fibres in frog's nerve, but since it has not been possible to work with single vertebrate fibres the local potentials have been studied mostly in crab or squid nerve.

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By following these changes in excitability and in local potential concurrently in single squid nerve fibres we have been able to show that the early divergence of the time course of excitability from that to be expected from the classical picture of the summation of electrotonic potentials can be explained as due to an additional contribution at the expense of the potential energy of the fibre itself. This contribution has been called the *local response* [Katz, 1937; Hodgkin, 1937]. Moreover, since the excitability curves which we have found for the squid are essentially similar to those deduced theoretically by Rushton [1937] and found for medullated fibres by Katz, the probability is greatly strengthened that the initiation of the nerve impulse is essentially similar in all nerves.

The method has been to study the changes in excitability at a particular point on the nerve fibre at various times after the application of cathodal and anodal conditioning stimuli, and to compare these changes directly with the form of the local responses following the shocks. In order to reduce the number of variable factors, we have confined our attention to the effects of "instantaneous" stimuli of a duration of about 25  $\mu$ sec.

#### DEFINITIONS

When a recording electrode is placed close to the electrode through which a conditioning stimulus is applied to a nerve fibre, potential changes of two types occur: (1) A *physical response* (polarization process, Hodgkin [1938], production and decay of electrotonic potential). This response is obtained whether the stimulus is cathodal or anodal. It outlasts the duration of the stimulus because the charge deposited on the membrane takes an appreciable time to leak away. (2) When the conditioning electrode is a cathode there develops as the stimulus approaches threshold a *physiological response* (local response, Hodgkin), viz. a potential change in addition to the physical response, and resulting therefore from local activation of the excitable mechanism of the fibre. The net local potential is then the resultant of these when both are present.

#### BIOLOGICAL TECHNIQUE

The animals used were *Loligo forbesi*, obtained at the Laboratory of the Marine Biological Station at Plymouth, to whose Director and Staff we are most grateful for their willing assistance.

Whole stellar nerves were dissected immediately after killing the animals, and after brief soaking in sea water were suspended in a moist chamber. The responses of the giant axons were usually examined with-

out dissecting the fibres from the nerve trunk, since dissection often leads to abnormal conditions in the fibre.

The moist chamber used was substantially identical with that described in the previous paper [Schmitt & Schmitt, 1940], with the single change that the vessel was suspended vertically. The nerve was thus suspended by its end and was only held to the electrodes by surface tension instead of lying over them. Platinized platinum electrodes were used throughout to minimize polarization at the electrodes.

### ELECTRIC TECHNIQUE

The cathode-ray oscillograph equipment and synchronizer used were essentially the same as those described by Schmitt & Schmitt [1940]. In order to improve the cathode-ray image a large (150 mm.) 3-anode cathode-ray tube was used with 2300 V. accelerating potential and a differentiator type automatic brilliancy control. This arrangement provided bright images in which the upstrokes of action potentials were kept of equal brilliancy with the cross lines, thus greatly facilitating photographic recording.

The stimulators were almost identical in design with the one previously used. The two stimulator units were independently powered, so as to permit reversal of stimulation polarity and to obtain complete independence of the strengths of the two stimuli.<sup>1</sup>

Independent synchronization controls were provided, so that the two shocks could be timed separately and either could precede the other in firing sequence. In experiments where both stimuli were fed through common leads, care was taken to prevent interaction of the two intensity controls. By the use of appropriate II and T section pads this interaction was reduced to approximately 1 % in the most extreme cases, this error being compensated for in the one or two cases where it could be detected. The intensity of stimulus from either stimulator, as indicated both by threshold measurements and by direct ballistic tests, was constant and reproducible to about 0.15 % over a short time, and calibrations remained constant to about 2 % during the whole series of experiments. As in the previous experiments, a long-range logarithmic dial gave the basic adjustment of shock intensity and a short proportional dial gave increments up to  $\pm 10$  %.

<sup>1</sup> The oscillographic equipment used in this research was generously loaned by the Department of Zoology, Cambridge University, and the Department of Physiology, University College, London.

Pure condenser discharge stimuli were used, except in a few cases where an earthed stimulating electrode was undesirable. Then the stimulator was coupled to the preparation by an air-cored transformer of low distributed capacitance (National Company OSR oscillator coil), or by a small iron-cored transformer.

The amplifier was of the cathode phase inversion type (Schmitt [1938]), push-pull throughout. With three stages of phase inversion, the differential ratio was approximately 7000:1 at all gains, even with large fractions of a volt input, so that stimulation artefact was negligible. When accurate measurement of the shock time was required, some current from the stimulator circuit was introduced directly into the amplifier.

### EXPERIMENTAL RESULTS

#### (1) *General course of the excitability changes and their relation to the local response*

If a fresh fibre in good condition is subjected to a cathodal sub-threshold conditioning shock, then the threshold near the point of application is lowered at all subsequent times at which any effect can be detected (Fig. 1 (a)) except for a possible very slight accommodative effect. However, in older preparations, and particularly if the fibre has been frequently and strongly stimulated, it can be observed that a just sub-threshold conditioning shock renders the fibre at first more but later *less* excitable than it was in the resting condition (Fig. 1 (b)). There is, therefore, in these latter preparations, a *local relative refractory period* following a conditioning shock.

In the preparations which show this local refractory period the local action potential following a just subthreshold conditioning shock diverges very widely from the form of the simple physical response. This indicates that partial excitation has occurred over a relatively great length of nerve without passing over into the propagated action potential. The subsequent decrease in excitability which is found to follow such a conditioning shock is therefore to be attributed to the presence of a refractory condition in the section of the fibre in which a local physiological response has occurred.

#### (2) *Refractory period of the local response*

Records such as those of Fig. 11 c-f show that there is indeed, in such fibres, a period after the application of a conditioning shock during which the test shock produces little or no physiological response from the fibre. There is, in fact, a *refractory period of the local response*.

The appearance of a period of lowered excitability following a sub-threshold conditioning shock therefore depends on a balance between the

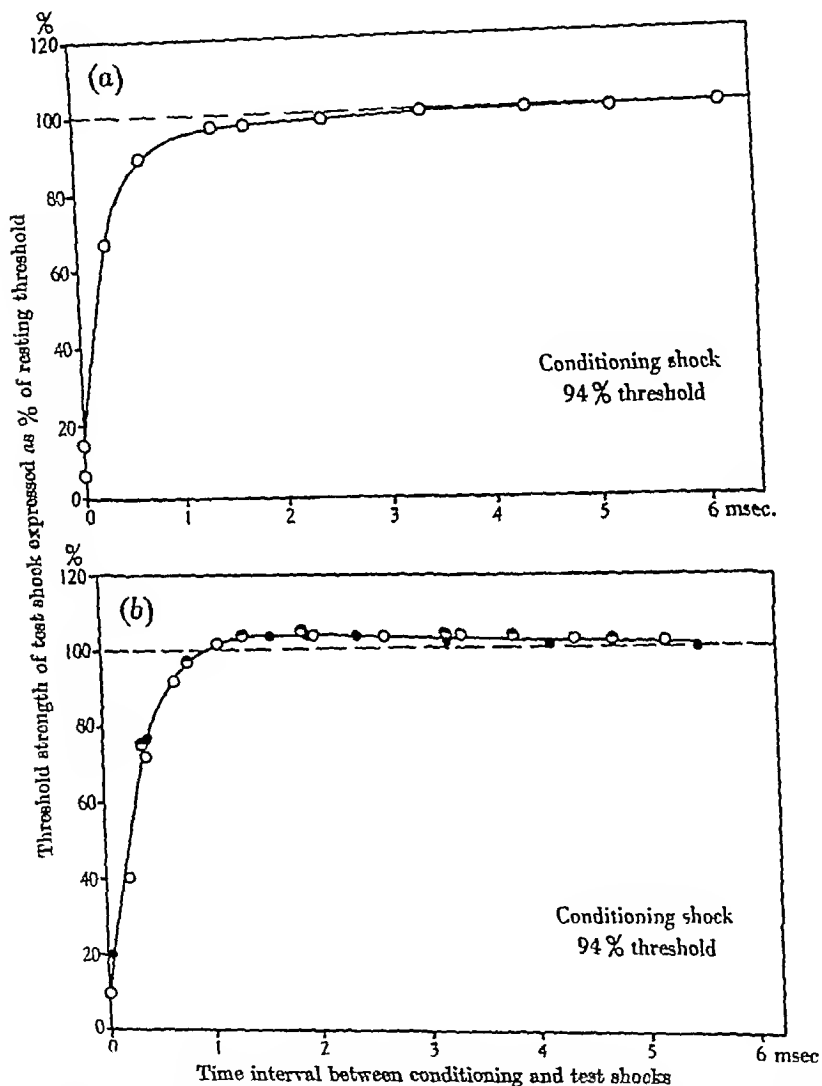


Fig. 1. Time course of excitability (a) in a fresh squid fibre, (b) in one showing a local relative refractory period. Three separate series of observations are plotted in (b) to indicate the reproducibility of the phenomenon.

safety ratio of the fibre [Schmitt & Schmitt, 1940], its length constant [Rushton, 1927, 1934] and the duration of the local refractory period. The

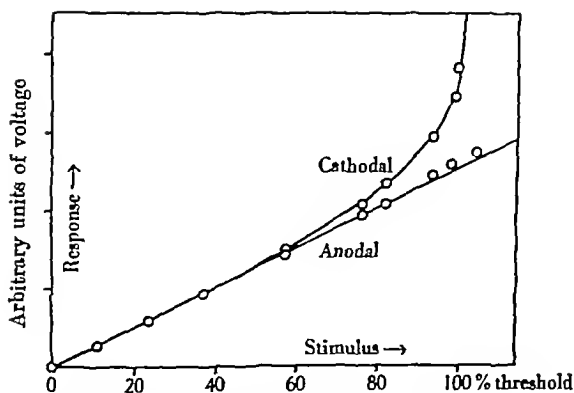


Fig. 2. Development of local potential. For anodal stimuli of all strengths the response is proportional to the stimulus. For cathodal stimuli this relationship applies only to stimuli of less than about half threshold. For near threshold stimuli the local potential rises rapidly.

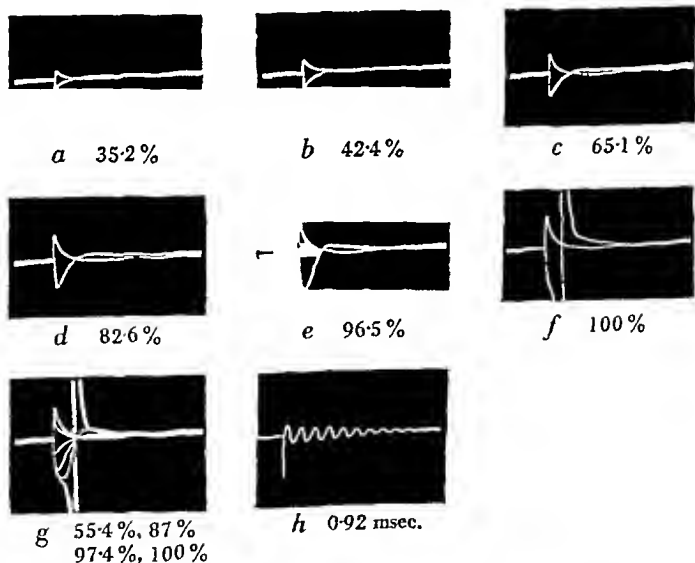


Fig. 3. Comparison of cathodal and anodal polarizations. Each record is a double exposure composed of the response of the nerve at the stimulating electrode to an anodal and to an equal cathodal stimulus. Percentages refer to strength of stimulus in terms of threshold. To stimuli of less than half threshold (*a* and *b*) there is no appreciable difference between the forms of anodal and cathodal responses, but with stronger stimuli (*c*, *d*, *e*, *f*) the cathodal response rises rapidly and assumes the characteristic form of the local action potential. In record *g*, the responses to four strengths of anodal and cathodal stimuli are superimposed. The 55% threshold stimuli give similar responses anodally and cathodally, but the 87, 97 and 100% shocks give rapidly increasing cathodal responses but practically superimposed ones anodally. The double cathodal responses for 100% are due to minute variations of threshold, each cathodal and anodal record being a double trace to check constancy.

local refractory period will appear when the safety ratio is low, and consequently a long region of nerve must be excited to produce enough physiological response to propagate. When such a nerve is conditioned with a stimulus nearly strong enough to excite, a long region is caused to give its physiological response. Once this region has ceased to be active and is still in its refractory period, a cathodal stimulus to be effective must now excite beyond this refractory region, and must consequently be of great strength.

On the other hand in a fresh fibre the safety ratio is high, so that a propagated impulse results from excitation of a very short length of nerve. A subthreshold shock, therefore, can only excite physiological response over a still shorter length, and the test shock can be made to reach out over this length without appreciable increment in strength. For example, in the nerve from which Fig. 2 was obtained there would be no appreciable hypoexcitability, since here a small fraction of an analytical unit of length can contribute enough local activity to propagate, and the total range of stimulus strength over which the purely physical response changes to the full-fledged action potential is barely enough to be noticeable when applied anodally under identical conditions (see Fig. 3).

The appearance of a period of lowered excitability following a conditioning shock is not necessarily dependent on any irreversible deterioration of the fibre. In the experiment shown in Fig. 4 the excitability cycle was first tested and the fibre then stimulated at 60 times threshold strength 100 times per sec. for 15 sec. On retesting 3 min. after this treatment it was found that a refractory state now followed the conditioning stimulus, and was so pronounced that a stimulus of more than three times the threshold intensity was required to stimulate when applied 1 msec. after a conditioning shock of 80 % threshold. Testing at subsequent times showed that the refractoriness then gradually passed away, until after half an hour the fibre had returned almost to its original condition, though a slight local relative refractory period could still be detected.

To determine the spatial distribution of this artificially induced local refractoriness, a nerve fibre was treated as in Fig. 4, but instead of allowing spontaneous recovery to occur, the local refractoriness in the vicinity of the tetanization was determined as quickly as possible. As is seen in Fig. 5, the refractoriness decreased on passing beyond the region to which the tetanus had been applied.

The condition of reduced excitability becomes more marked as the conditioning stimulus approaches threshold strength (Fig. 6). There is,



however, usually a certain critical time after stimulation at which no effect on the excitability is produced, whatever the strength of the conditioning stimulus. Thus in Fig. 6 a test stimulus placed at 1.2 msec.

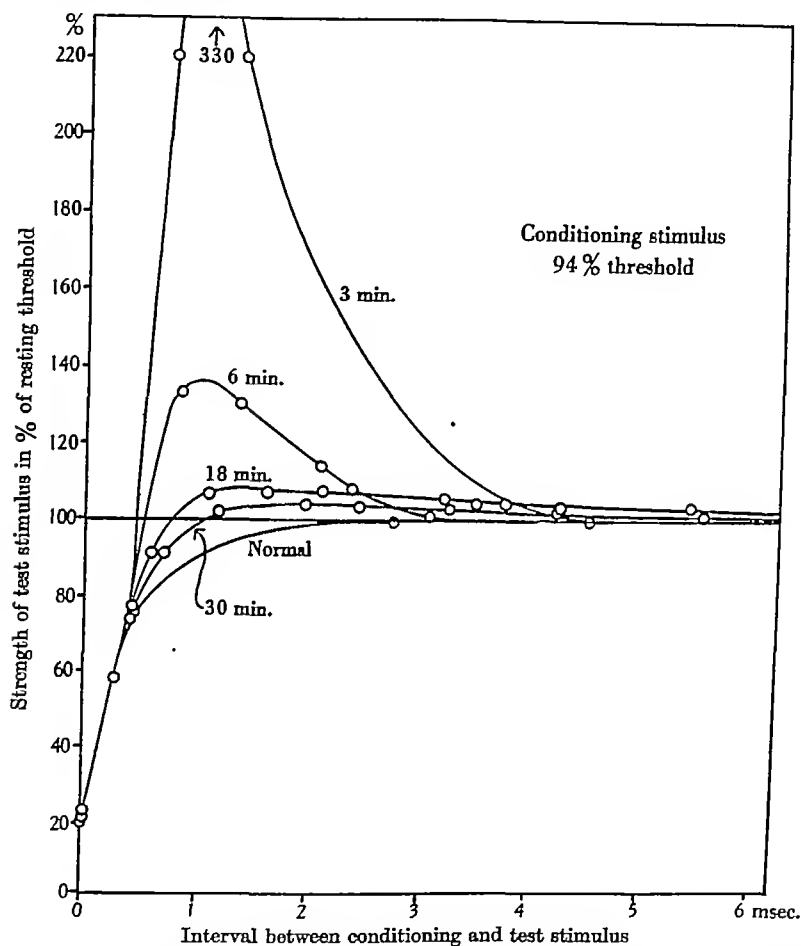


Fig. 4. Local refractory period (temporal variation). The excitability curve of a normal squid axon was first tested and found to be as in the curve marked "normal". The fibre was then stimulated at 60 times threshold strength, 100 times a second for 15 sec. and the excitability curves taken again 3, 6, 18 and 30 min. afterwards. The local refractory period is at first very pronounced but the nerve gradually recovers until it reaches almost to the original condition.

finds a constant threshold despite a wide variation in the strength of the conditioning shock. This apparently implies that the state of reduced excitability, whatever its nature, runs its course in close step with the

excitatory process, since a mere fortuitous summation of processes of different time courses would almost inevitably lead to a shift in the moment of unit excitability with alteration in the intensity of the conditioning shock.

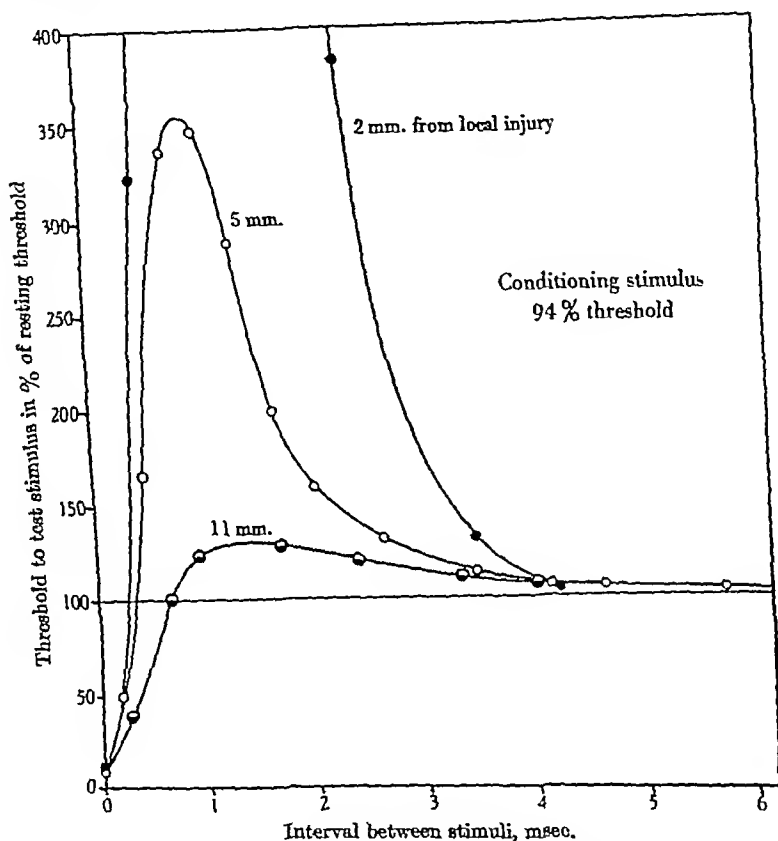


Fig. 5. Local refractory period (spatial variation). After the safety ratio of a nerve has been reduced locally by overstimulation, the local refractoriness becomes very marked in the vicinity of the injury and decreases gradually as more remote points are tested. Two mm. from the point of injury the threshold to a test shock remained more than 4.5 times the resting threshold from 0.5 until 2.5 msec. after a 94 % conditioning stimulus.

The wide variation of the local response with change in the strength of the conditioning stimulus is illustrated in Fig. 7. Here two cathodal impulses were applied at a little longer than the critical interval (almost exactly 1 msec. in this case), and the test stimulus set to just threshold strength, with the resultant propagated action potential of record  $\alpha$ . As

the conditioning stimulus was increased in the records *b-e* the local response to the second stimulus was seen to decrease progressively as physiological response is taken over by the conditioning stimulus, although the net total local response remains approximately constant. At *f* an action potential is seen to occur, but by now the conditioning stimulus has reached threshold, and is adequate to excite on its own.

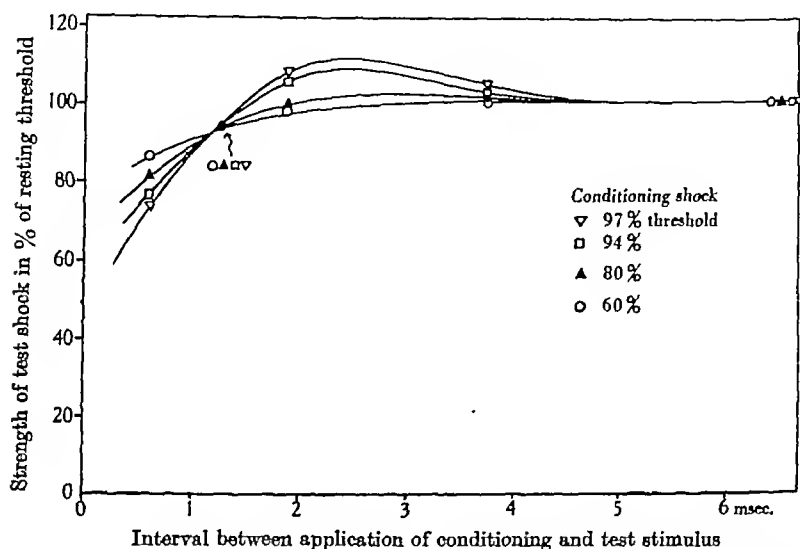


Fig. 6. In axons with low safety ratio, a time interval is often found at which any subthreshold conditioning stimulus has very little effect on the threshold for the test stimulus. In this case the interval was about 1.2 msec.

Record *g* is included to show the local response in the refractory period following a *propagated* action potential. It will be seen by comparison with record *e* that the second response is similarly depressed by an adequate and by a just subthreshold conditioning stimulus.

### (3) Other changes of excitability at long periods after a conditioning shock

Following the local refractory period after a subthreshold conditioning shock (or the return to unit excitability if no refractory period is detectable), there is often a second period of raised excitability. This *local supernormal period* may last for several tenths of a second, but the change of threshold is only very small, and the phenomenon has not been further investigated during the present work.

Another slow change in excitability which can be observed is a hyperexcitability 1-20 msec. after an *anodal* conditioning shock. This is partly associated with the slowly disappearing back polarization produced by the stimulus (accommodation, see p. 64 and Fig. 11e), and is

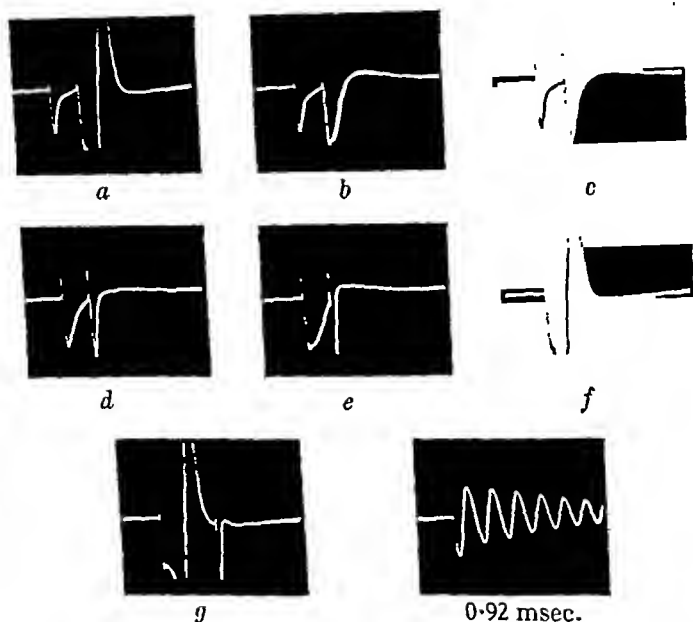


Fig. 7. Local action potential at unit excitability point. Two stimuli are applied to a nerve with low safety ratio at about the time interval for unit excitability (ca. 1.2 msec. in Fig. 6, 1.0 msec. in this nerve). The test shock is made just threshold and the conditioning shock varied from well below to just threshold. With weak conditioning (a and b) there is only a physical response to the conditioning stimulus, but the large local physiological response to the test stimulus causes (a) or nearly causes (b) excitation. With progressively stronger conditioning stimuli (c, d, e), the conditioner elicits an increasingly large local physiological response, and the test shock falling in the local refractory period can contribute only physical response. In f a response occurs but only because the conditioner has now reached threshold strength. Record g is included to permit direct comparison of the local response during the local refractory period in records a-f with the local response during the refractory period following adequate stimulation.

partly an electrode phenomenon, for it can be reduced by the use of separate electrodes for test and conditioning stimuli, or by careful blacking of common electrodes.

(4) *Excitability changes during the early summation period*

In order to make the knowledge of the process of excitation in the squid comparable with that revealed by the investigations of Gasser, Erlanger, Katz and others on frog and *Carcinus* nerves and other tissues it was essential to make an especially careful survey of the summation effects of two shocks delivered less than 1 msec. apart. A parametric plotting scheme similar to that used by Katz [1937] was employed, but it was felt that no advantage was gained by introducing the abstract "amount of excitatory state". Instead, the directly measured intensity of stimulation was plotted, this quantity being taken as proportional to the peak potential of the stimulating impulse, which was maintained of constant potential wave form.

The results of this survey are given in Fig. 8. The curves in the upper section refer to experiments with subthreshold conditioning shocks of the fractional strength indicated on each curve, while those below refer to experiments with superthreshold conditioning shocks followed by anodal test shocks. Any one curve then gives the strength (anodal or cathodal) of a test shock which must be applied at the time given by the abscissa after the conditioning shock to just bring about or just fail to bring about excitation of a propagated impulse. In all cases, the pairs of stimuli were applied in a regular sequence about once per second, so that slow changes of excitability would be rendered unimportant. Before recording a strength a setting was found at which the preparation always just produced or just failed to produce a propagated impulse, thus avoiding the considerable changes of threshold sometimes brought about by excitation itself. Experiments with anodal conditioning shocks are not plotted as they are of less interest, although the behaviour in this case can be inferred from the curves shown later for spatially separated stimulation (Fig. 12).

It will be observed that in all cases where a conditioning shock less than about half threshold was used, summation decreased in an approximately exponential way, as would be predicted from the electrical theory of leaky cables. With conditioning shocks of more than 50 % but less than threshold strength, this exponential decay gradually became more and more modified, until by 90 % of threshold a prolonged convexity became evident, indicating a considerable retention of activity which lasts for nearly half a millisecond.

With superthreshold conditioning stimuli it was found that shocks only slightly in excess of threshold could be prevented from causing a

propagated wave by anodal shocks applied during the first half millisecond, the shock strength necessary to prevent propagation increasing rapidly with the interval between conditioning and test shock. With

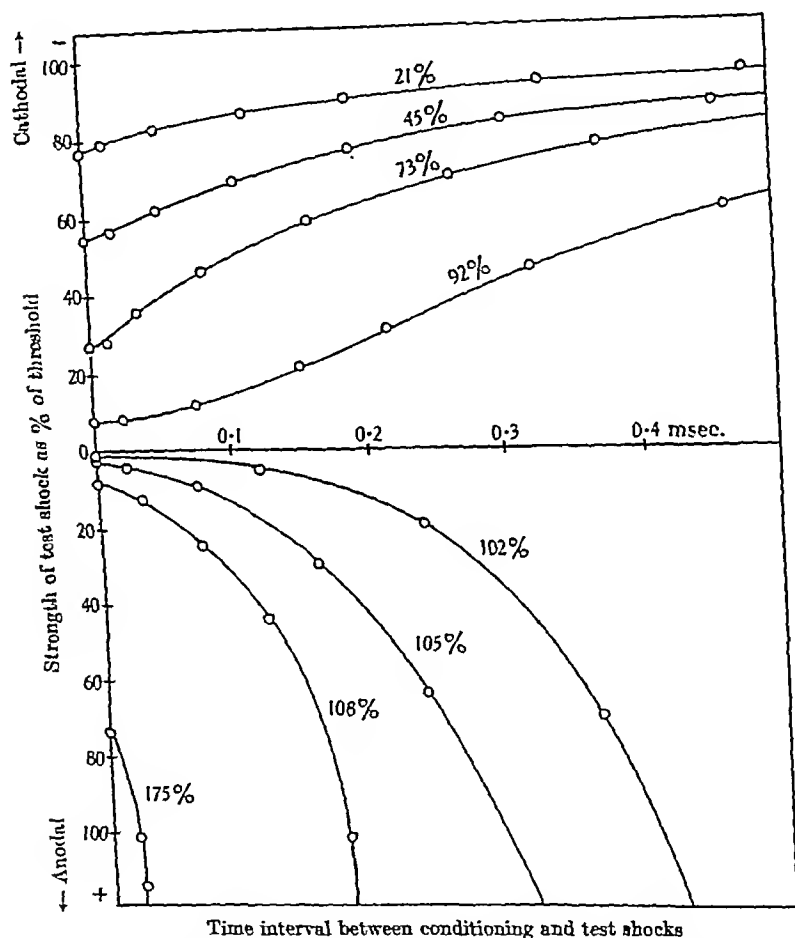


Fig. 8. Excitability of squid giant axon. The percentages marked beside the several curves refer to the strength of the cathodal conditioning stimulus relative to resting threshold. Ordinates indicate the strength of test shock which will just allow an impulse to propagate. Negative ordinates indicate in terms of threshold for cathodal stimuli the anodal stimulus required to prevent propagation.

shocks stronger than about 1.5 times threshold an impulse could be inhibited only during the first few microseconds after application of the conditioning shock, and then only by very strong anodal test shocks. It

must also be remembered that for times shorter than  $25\mu\text{sec.}$  the two stimuli overlapped, and thus tended to cancel electrically.

To permit direct comparison of these results with those obtained by Katz on frog nerve, his data have been re-plotted on the same coordinates

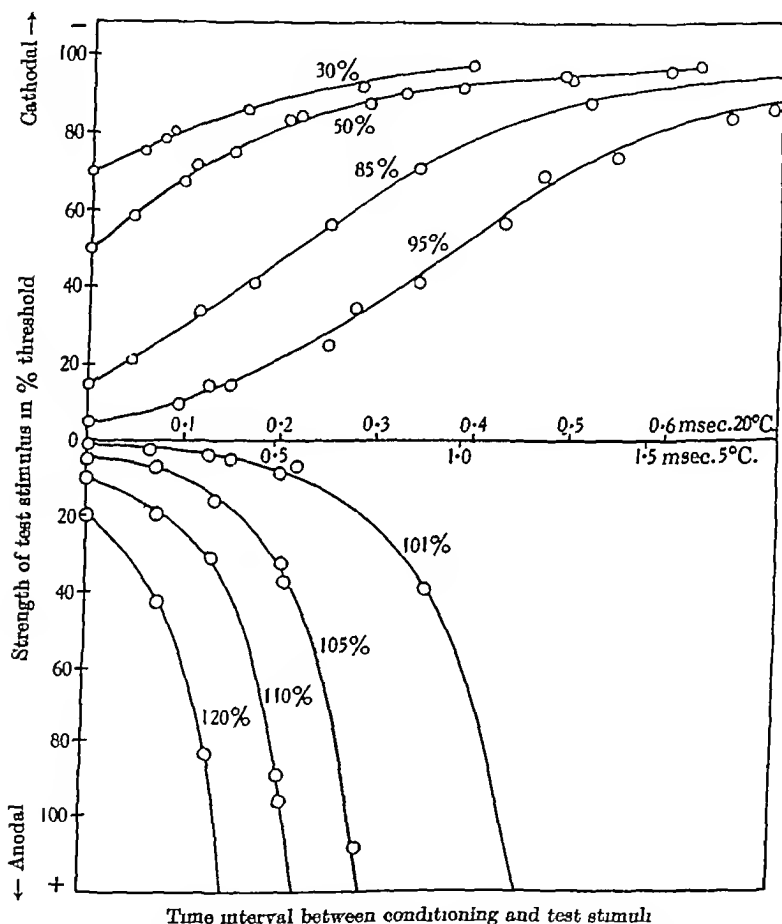


Fig. 9. Excitability of frog sciatic nerve Data of Katz [1937] re plotted to allow direct comparison with Fig. 8

as ours in Fig. 9. The similarity is unmistakable. It is interesting that the absolute time constants are very nearly equal for both tissues, a result which might be expected from the similarity of the velocities of propagation and length constants in the two cases.

(5) *Potential changes during the early summation period*

To coordinate the excitability data with the local responses, series of records such as that shown in Fig. 10 were obtained. First it must be noted that the local potential produced by a just subthreshold stimulus lasts for approximately 1 msec. (Fig. 10*a*), which is about the period

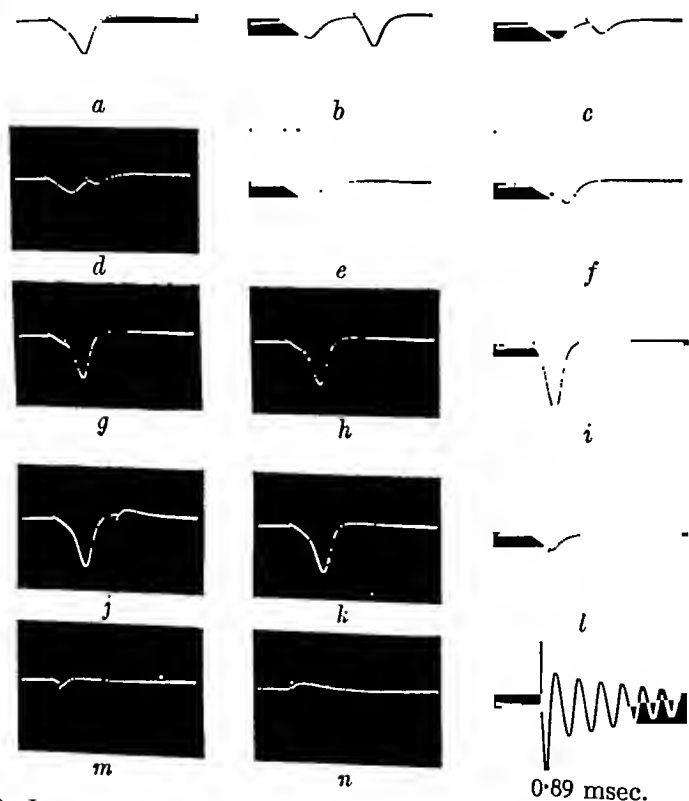


Fig. 10. Local action potential summation recorded at a distance from stimulating electrode. Record *a* shows the response to a single just subthreshold cathodal shock. In records *b* to *i*, two approximately equal, just subthreshold cathodal stimuli are applied at varying time intervals and the resulting potentials recorded a little distance away. As the two stimuli approach in time the local response to the second first decreases but then becomes greater than the sum of the two original potentials. In records *j* to *n*, the second stimulus is reversed (anodal) and its effect is shown merely to subtract physically from the response except when it follows the cathodal pulse very closely and thus prevents the spread of local physiological activity near the stimulating electrodes and decreases the response markedly. The threshold rose slowly during this series and the cathodal stimulus was slightly increased at record *j*.



during which the threshold for a second shock is found to be lowered. The series *b-l* of Fig. 10 shows the behaviour of the local potential recorded at a short distance from the stimulating electrode when two slightly sub-threshold shocks of approximately equal strengths are applied at various time intervals. With a long interval between the shocks in record *b*, a pair of completely separated typical (physiological) responses occurs. As the second impulse approaches closer in time to the first in records *c* and *d*, the second local response is seen to diminish markedly and to change shape towards the condition found in record *e*, which can be shown by comparison with the effect of an anodal stimulus of similar strength (*j*) to represent the physical response alone.

In record *f* of Fig. 10 we come to the steep part of the summation region of Fig. 8, and correspondingly the second stimulus occurs while the local response to the first stimulus is still developing. Under these conditions the second stimulus evokes additional local response, which becomes greater and greater in *g* and *h*, and in *i* is distinctly greater than the sum of the original two local responses. This again is what would be expected from the non-linear relation between stimulus strength and magnitude of the net local response (see Fig. 2). It should be remembered that the time courses of changes of excitability and of accompanying local activity would not be expected either on theoretical or on experimental grounds [Katz & Schmitt, 1940] to correspond identically.

The records *j-n* correspond to points in the lower half of Fig. 8 and illustrate the summation of a just subthreshold cathodal stimulus with an anodal impulse of equal strength subsequently applied. As would be anticipated from Fig. 8, anodal impulses applied after the interval when summation is nearly perfect have little or no effect on the response (records *j* and *k*). During the early part of the summation period, an anodal pulse cannot remove the local physiological activity already started by the cathodal impulse, but prevents this local activity from exciting further regions. If the anodal stimulus is applied before the cathodal, or before the local response has got beyond the "physical" state, however (record *n*), the spread of local activity can be stopped completely even though the conditioning shock be considerably above threshold.

The events which accompany local summation and anti-summation of two equal stimuli at the stimulating electrode are illustrated in Fig. 11. In record *a* a propagated response is obtained to the test stimulus because the latter falls in the supernormal period. In *b* the test stimulus falls in a period of slight depression of excitability. Records *c-f* again show, as in Fig. 10, the gradual loss of physiological local response to a

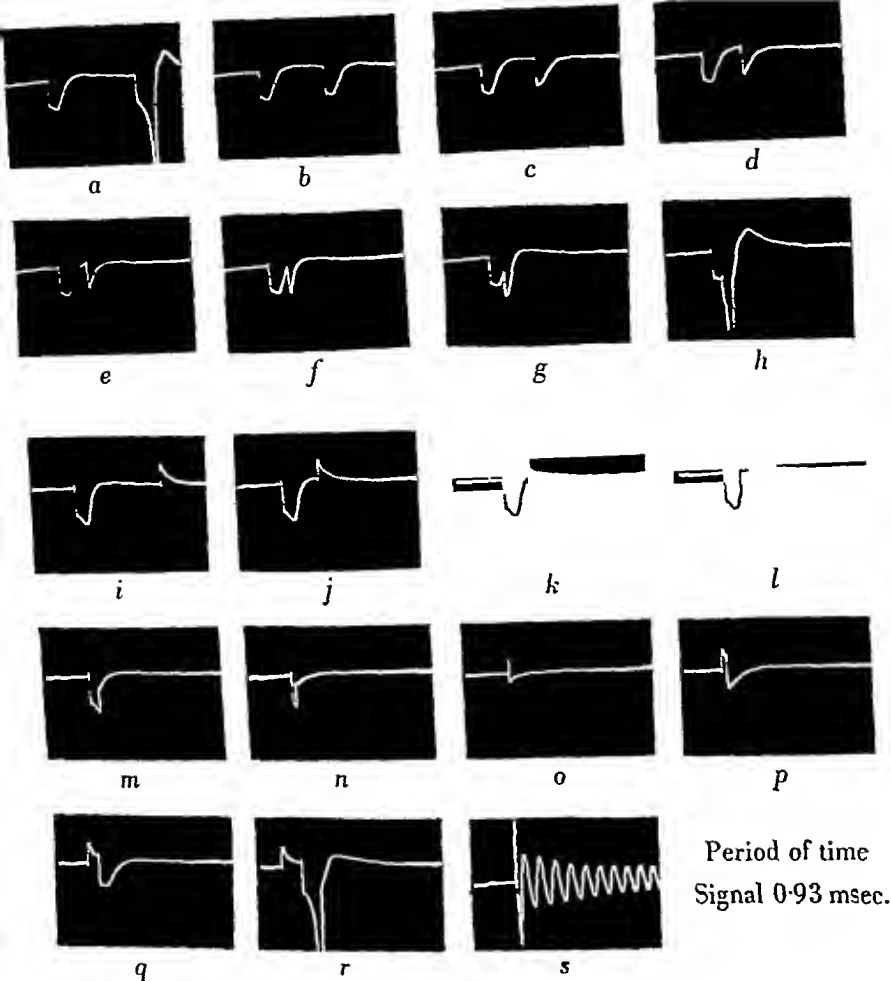


Fig. 11. Local action potential summation near stimulating electrode. In records *a-h* two approximately equal, just subthreshold cathodal stimuli are applied to the nerve through a common electrode at varying time intervals and the resulting potentials recorded within 0.5 mm. of the stimulating electrode. At the long interval of *a*, the test stimulus finds the tissue supernormal so that a propagated response is initiated. As the interval is progressively decreased in *b, c, d*, and *e*, the test stimulus comes more and more into the local refractory period and consequently loses local physiological activity and assumes the form of the purely physical response. In *f, g*, and *h* the test stimulus is in the summation interval and so sums physically with the conditioning stimulus, resulting in a propagated response in *h*. In records *i-r* the conditioner is a cathodal shock of just subthreshold strength but the test shock is anodal. While the anodal impulse is outside the summation interval (*i, j, k, l*) the response is merely the algebraic sum of the two impulses separately. During the summation interval (*m* and *n*) the anodal impulse not only subtracts physically from the local potential present, but also prevents the development of further local physiological activity after its application. In *o* and *p* it precedes the cathodal impulse and no local physiological activity starts. Applied still earlier the anodal shock may have no effect (*q*), due to equal summation and accommodative effects, or if early enough it may facilitate the cathodal impulse sufficiently to yield a response (*r*).

during which the threshold for a second shock is found to be lowered. The series *b-l* of Fig. 10 shows the behaviour of the local potential *recorded at a short distance from the stimulating electrode* when two slightly subthreshold shocks of approximately equal strengths are applied at various time intervals. With a long interval between the shocks in record *b*, a pair of completely separated typical (physiological) responses occurs. As the second impulse approaches closer in time to the first in records *c* and *d*, the second local response is seen to diminish markedly and to change shape towards the condition found in record *e*, which can be shown by comparison with the effect of an anodal stimulus of similar strength (*j*) to represent the physical response alone.

In record *f* of Fig. 10 we come to the steep part of the summation region of Fig. 8, and correspondingly the second stimulus occurs while the local response to the first stimulus is still developing. Under these conditions the second stimulus evokes an additional local response, which becomes greater and greater in *g* and *h*, and in *i* is distinctly greater than the sum of the original two local responses. This again is what would be expected from the non-linear relation between stimulus strength and the magnitude of the net local response (see Fig. 2). It should be remembered that the time courses of changes of excitability and of accompanying local activity would not be expected either on theoretical or on experimental grounds [Katz & Schmitt, 1940] to correspond identically.

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The events which accompany local summation and anti-summation of two equal stimuli at the stimulating electrode are illustrated in Fig. 1. In record *a* a propagated response is obtained to the test stimulus because the latter falls in the supernormal period. In *b* the test stimulus falls in a period of slight depression of excitability. Records *c-f* again show, as in Fig. 10, the gradual loss of physiological local response to

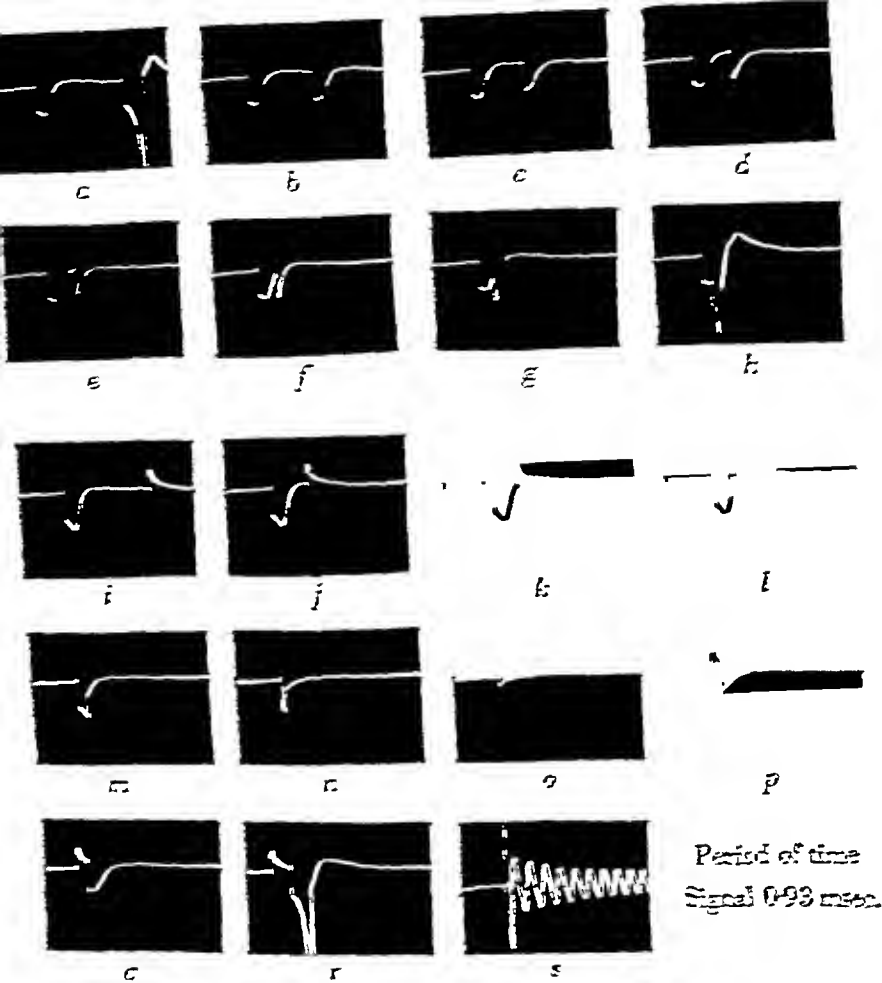


Fig. 11. Local action potential summation near stimulating electrode. In records a-h two approximately equal just subthreshold cathodal stimuli are applied to the nerve through a common electrode at varying time intervals and the resulting potentials recorded within 0.2 mm. of the stimulating electrode. At the long interval of a, the test stimulus falls just above threshold so that a propagated response is initiated. As the interval is progressively decreased in b, c, d, and e the test stimulus comes more and more into the local refractory period and consequently loses local physiological activity and assumes the form of the purely physical response. In f, g, and h the test stimulus is in the summation interval and so sums physically with the conditioning stimulus resulting in a propagated response in h. In records i-l the conditioning is a cathodal shock of just subthreshold strength but the test shock is anodal. While the anodal impulse is outside the summation interval (i, j, k, l), the response is merely the algebraic sum of the two impulses separately. During the summation interval (m and n) the anodal impulse not only subtracts physically from the local potential present, but also prevents the development of further local physiological activity after its application. In o and p it precedes the cathodal impulse and no local physiological activity occurs. Applied still earlier the anodal shock may have no effect (q), due to equal summation and accommodative effects, or if early enough it may facilitate the cathodal impulse sufficiently to yield a response (r).

during which the threshold for a second shock is found to be lowered. The series *b-l* of Fig. 10 shows the behaviour of the local potential recorded at a short distance from the stimulating electrode when two slightly sub-threshold shocks of approximately equal strengths are applied at various time intervals. With a long interval between the shocks in record *b*, a pair of completely separated typical (physiological) responses occurs. As the second impulse approaches closer in time to the first in records *c* and *d*, the second local response is seen to diminish markedly and to change shape towards the condition found in record *e*, which can be shown by comparison with the effect of an anodal stimulus of similar strength (*j*) to represent the physical response alone.

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The events which accompany local summation and anti-summation of two equal stimuli at the stimulating electrode are illustrated in Fig. 11. In record *a* a propagated response is obtained to the test stimulus because the latter falls in the supernormal period. In *b* the test stimulus falls in a period of slight depression of excitability. Records *c-f* again show, as in Fig. 10, the gradual loss of physiological local response to a

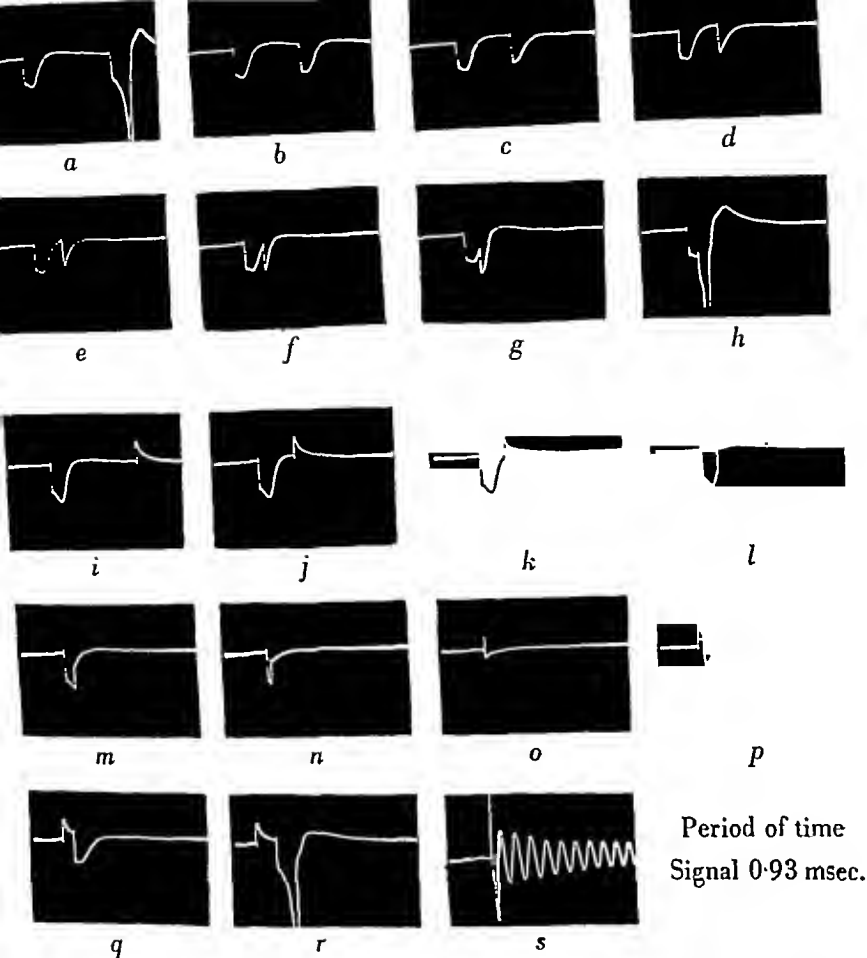


Fig. 11. Local action potential summation near stimulating electrode. In records *a-h* two approximately equal, just subthreshold cathodal stimuli are applied to the nerve through a common electrode at varying time intervals and the resulting potentials recorded within 0.5 mm. of the stimulating electrode. At the long interval of *a*, the test stimulus finds the tissue supernormal so that a propagated response is initiated. As the interval is progressively decreased in *b, c, d*, and *e*, the test stimulus comes more and more into the local refractory period and consequently loses local physiological activity and assumes the form of the purely physical response. In *f, g*, and *h* the test stimulus is in the summation interval and so sums physically with the conditioning stimulus, resulting in a propagated response in *h*. In records *i-r* the conditioner is a cathodal shock of just subthreshold strength but the test shock is anodal. While the anodal impulse is outside the summation interval (*i, j, k, l*) the response is merely the algebraic sum of the two impulses separately. During the summation interval (*m* and *n*) the anodal impulse not only subtracts physically from the local potential present, but also prevents the development of further local physiological activity after its application. In *o* and *p* it precedes the cathodal impulse and no local physiological activity starts. Applied still earlier the anodal shock may have no effect (*q*), due to equal summation and accommodative effects, or if early enough it may facilitate the cathodal impulse sufficiently to yield a response (*r*).



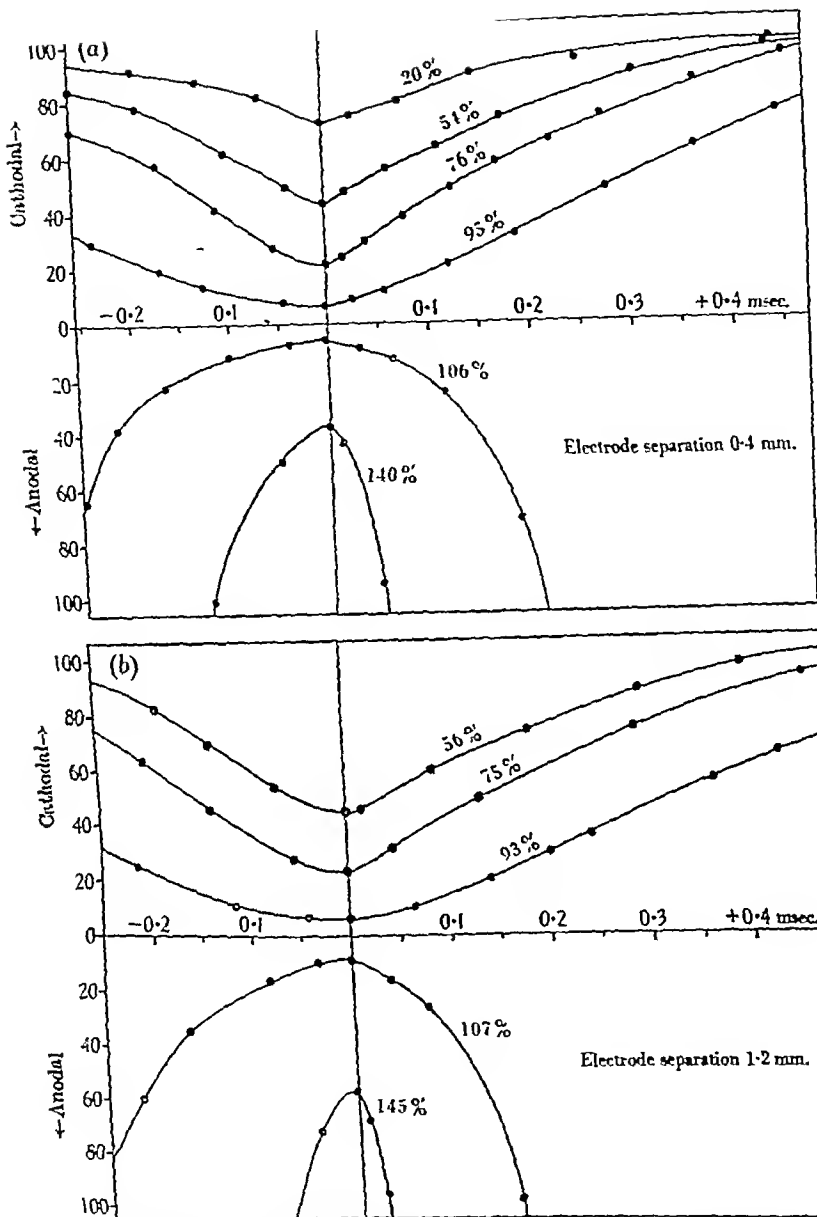


Fig. 12. Summation of spatially separated stimuli. The conditioning stimulus is applied at a point 0.4 mm. in (a) and 1.2 mm. in (b) from the point tested. The conditioning stimulus was always cathodal and of the strengths indicated beside each curve in per cent of threshold. Negative abscissae refer to experiments in which the "test" shock preceded the "conditioning" stimulus. Negative ordinates refer to experiments with superthreshold conditioning where the response was just inhibited by an anodal test stimulus. The asymmetry of the curves about the Y axis indicates the relatively greater persistence of activity after a just subthreshold shock than after a lesser one. The anodal curves illustrate the much greater ease with which an impulse can be prevented before it has developed than blocked after it has developed.



In these last two experiments readings were taken with the test shock preceding as well as succeeding the conditioning stimulus. In the graphs points on the left of the origin (time negative) are for "test" shocks preceding the conditioning stimulus so that the effect of anodal conditioning shocks is indicated. As might be expected, an anodal impulse is much more effective in preventing excitation than in stopping it. The time scale of the figure is too open to show the accommodation effect of the anodal shocks.

#### (6) *Correlation of excitability and potential changes*

Summarizing the results, it is seen that weak (i.e. less than about 50 % of threshold) cathodal stimuli leave the nerve in a supernormal state which passes away approximately exponentially, and is accompanied by a local polarization potential (physical response) which dies away in approximately the same time and in the same way as the increased excitability. With such weak strengths of conditioning shock the response evoked by the test shock when it is just subthreshold shows a physiological response which starts on the tail of the (physical) response from the conditioning stimulus.

Strong subthreshold cathodal stimuli (i.e. 50–100 % of threshold) are followed by a period of hyperexcitability lasting longer and of different time course from that which would be expected on purely physical bases. With such intensities of conditioning stimulation the conditioning shock is accompanied by a large local potential with a non-exponential time course of nearly the same shape as that of the heightened excitability. With conditioning stimuli of around 60–70 % of threshold strength, both the test and the conditioning stimuli are accompanied by "physiological" response type polarizations, whatever the time interval between them, but in cases where 90–100 % of threshold conditioning shocks are used, the test shock applied during the local refractory period is not followed by a "physiological" local response, but only by a physical response. During the early portion of the summation period, therefore, the second stimulus is unable to elicit any local physiological response. Since, none the less, the fibre is still hyperexcitable at this time, it must be that the final thrust which sets off the propagation is the electrical stimulus itself, adding to what is left of the local response from the first stimulus, not itself producing physiological local response.

With superthreshold conditioning stimuli, the local potential recorded at the stimulating electrodes is either of the physiological response shape or of the "instantaneous" shape which results from stimulation intense

enough to set the nerve into instantaneous propagation without a preliminary millimetre or two of incremental conduction (see Fig. 13). In the slightly superthreshold cases, an anodal stimulus strong enough to prevent propagation is seen not only to cause a negative exponential shift of the pattern, but also to arrest instantaneously the further development of physiological local potential. In nicely adjusted cases where an anodal test shock just insufficient to prevent propagation is given, the already developing local potential is seen to pause briefly and then re-develop.

In the cases where an anodal conditioning shock is given, there is no evidence that it does more than produce a polarization, the remnant of which a subsequent cathodal impulse must overcome. Due to accommodation this remnant may under suitable conditions become negative.

### DISCUSSION

*From the point by point correlation which has been established between the development of local action potential and the onset of excitation under widely divergent conditions of stimulation with pairs of variously polarized stimuli it must be concluded, at least in the case of squid nerve, that excitation results from the simple electric summation of local physiological action currents with appropriate components of externally applied stimuli. That a similar rule applies in the case of medullated nerves has not yet been established experimentally, but is almost certain from the identity of the excitability curves for medullated and non-medullated nerves, such as those obtained by Katz and in the present work (Figs. 8 and 9).*

It would appear that each element of nerve, upon being adequately stimulated, undergoes excitation and makes its contribution to the local action potential quite independently of whether a propagated impulse exists or is being initiated in the nerve or not. This contribution runs a time course which may be deduced from Fig. 13. This time course is not that of the propagated action potential, which depends largely upon the safety ratio and length constant of the nerve, but is considerably quicker.

It appears probable that the inhibiting action of anodal stimulation is due to electrical neutralization of local action currents rather than to restoration of the excited regions of the nerve to the normal condition. It is therefore much easier to prevent the development of propagation in response to an adequate cathodal stimulus by an anodal impulse preceding the cathodal than by one succeeding it, as the preceding anodal stimulus need only balance the applied cathodal stimulus while the

anodal stimulus following a cathodal must neutralize both the cathodal stimulus and the local action currents which it has evoked. As the local activity spreads to regions farther and farther from the point of stimulation, this becomes increasingly difficult as the anodal stimulus must not only neutralize more and more local current but must also reach out farther from the point of stimulation.

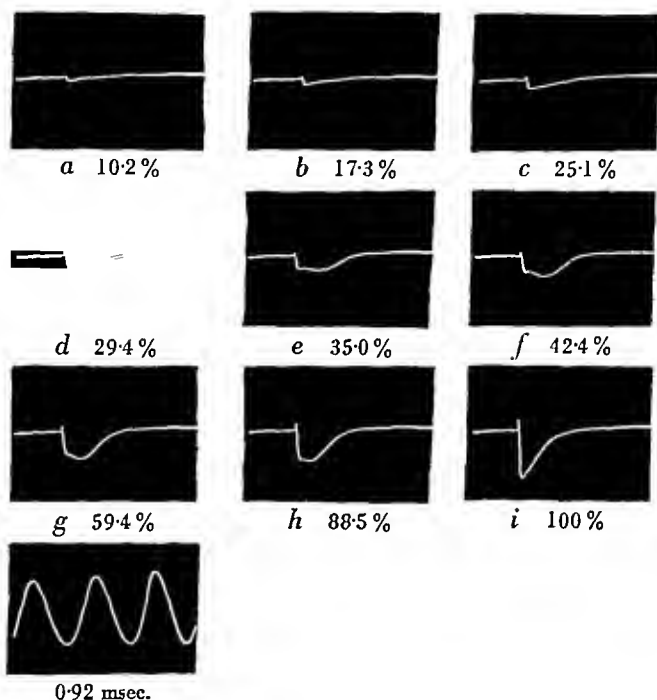


Fig. 13. Development of local action potential. The nerve is allowed to reach a state of reduced safety ratio and the potential developed at a point 0.5 mm. from the stimulating electrode recorded. With weak stimuli the potential is the physical response due to the electrotonic spread of stimulus, and consequently falls off nearly exponentially (*a, b, c*). Then in a short range of stimulus strength, the local physiological response develops to its maximum (*d-g*). With further increases in stimulus strength, little further physiological response is produced, but only increased electrotonic spread of stimulus (compare with response in nerve with high safety ratio, Fig. 3). The percentages give the relative strengths of stimuli in terms of the strongest.

It is not yet possible to say definitely whether the degree of activity in any element of nerve is graded or not, as the length of nerve excited and the degree of excitation of any one element are, as yet, inextricably bound up together. It may be possible through the use of a properly

shaped electric stimulating pattern to bring a considerable length of nerve into a constant state of excitation and thus learn whether the net threshold is sharp or diffuse.

The familiar short-time variations of excitability brought about by the application to a nerve of anodal or cathodal conditioning stimuli [Katz, 1937] can be described in quite simple terms if one is prepared to consider nerve as a polarizable electric cable which becomes excited and contributes energy at any point where stimulation has exceeded the minimum set by the excitation laws [Hill, 1936]. Any point which is thus excited contributes its local activity and then undergoes an absolute and perhaps a relative refractory period.

Consider, for example, the case of a quick anodal conditioning stimulus. This stimulus, whatever its strength, results in the passage of a damped anodal pulse progressing away from the source, followed by a small cathodal remainder due to polarization. A cathodal pulse of even slightly superthreshold strength preceding the anodal will very soon have recruited enough local activity to start excitation in regions several analytical units of length away from the site of stimulation, and hence out of reach of the rapidly attenuating anodal pulse. It is thus very difficult to stop an impulse with an anodal pulse unless the impulse is still in the immediate vicinity and not yet full grown.

An anodal impulse applied before the same cathodal one would fare much better, since now it would have only to balance the direct physical catelectrotonus, without which no local activity would be generated. This effect is clearly shown by the asymmetry of the anodal branches in Fig. 12 (a) and (b). With the anodal conditioning impulse occurring still earlier, a cathodal remnant would linger and the post anodal enhancement, an accommodation effect, would occur, as seen in Fig. 11, records *q* and *r*.

With weak cathodal conditioning pulses the reverse of the anodal process occurs, and there is first a physical summation period and later a very small accommodative rise of threshold which is barely observable.

The early summation period represents simply the interval during which the physical response, plus the physiological response which it has excited, are in being and can assist the second stimulus. Where the greater part of the response is contributed by the stimulus, the resultant hyperirritability dies away nearly exponentially. So soon, however, as much of the hyperexcitability is contributed by local physiological activity, its effect persists longer (1) because of the finite duration of the local activity and (2) because a decremental impulse is set up which tends to

excite more distant loci at later times, they in turn summing with the second stimulus. The portion of the nerve which contributes the local physiological activity soon enters its local refractory state, and if the safety ratio is low there follows a post-cathodal depression.

The variations of excitability following an adequate stimulus are easily correlated with the variations of local activity, for immediately after an impulse has been excited, all regions in the vicinity of the stimulus are refractory and then gradually recover from the point of stimulation outward. During the early stages of recovery the safety ratio is severely reduced and consequently a great length of nerve must be excited to produce propagation. Gradually recovery occurs and the threshold drops as a shorter and shorter length suffices to excite.

The form of the recovery curve following an adequate shock is not exactly that obtained with subthreshold conditioning stimuli, because in one case only regions immediately in the vicinity of the electrode have been rendered refractory, while in the other the whole length of the nerve is depressed practically simultaneously. There is therefore much greater depression than that produced locally by subthreshold conditioning, after which only a minute region outside the depressed area need be excited to bring about propagation. As would be expected, the similarity between the recovery curves for subthreshold and for adequate stimulation becomes greater as the safety ratio of the nerve is reduced.

Thus the variations of local potential and the accompanying changes of excitability which follow various sequences of conditioning shocks can be understood, without making any specific assumptions about the nature of the excitatory process, by comparison of the nerve fibre to a polarizable electric cable capable of yielding a certain pattern of electrical energy at any point which is suitably polarized cathodally. This study, however, fully supports the view that the local physiological response produced by subthreshold cathodal stimulation is an activity in every way similar to that by which each region of a nerve fibre stimulates its neighbours during the propagation of a normal impulse.

#### SUMMARY

1. A new technique is described for the quick and accurate determination of nerve excitability. Two short shocks of separately adjustable strengths and polarities are used, both shocks being electrically synchronized with the cathode-ray time base at any desired time interval.

2. In single nerve fibres of the squid (*Loligo*), the variations of excitability which result from conditioning the fibre with various subthreshold

strengths of cathodal and anodal stimuli were correlated with the variations of local action potential occasioned by the conditioning stimuli.

3. Thus cathodal conditioning stimuli of less than half threshold strength are associated with exponentially decreasing (*physical*) local potentials and with exponentially decreasing extra excitability of similar time course and proportional magnitude.

4. Near threshold conditioning stimuli bring about disproportionately prolonged (*physiological*) local action potentials and correspondingly prolonged extra excitability of similar time course.

5. From the correlations thus made between variations of excitability and development of local action potential under various conditions it must be concluded that excitation in the squid fibre results from the electrical summation of appropriate components of local action currents and of the applied stimulus. This implies that propagation results from the spread of current produced by local physiological activity.

6. That these conclusions apply also to medullated fibres is made probable by the very close resemblance of the whole family of excitability curves found in the present investigation of single squid fibres to those of Katz found for bundles of frog sciatic fibres.

7. From the diminution of physiological local response to a cathodal stimulus applied to a nerve fibre shortly (*ca.* 1 msec.) after a just subthreshold conditioning stimulus, it is concluded that there exists a *refractory period of local response*.

8. In a fresh nerve fibre with high safety ratio this refractory period does not lead to the explicit appearance of a post-cathodal depression. If, however, the fibre has a low safety ratio, so that a relatively long section of it can be excited into local physiological activity without arousing a propagated response, then a marked period of lowered excitability appears after a subthreshold cathodal stimulus.

9. This local relative refractory period can be artificially induced in a fresh fibre by brief intense tetanization. It is then found to occur only in the region close to the tetanizing electrodes and to be most pronounced immediately after the tetanus, the fibre later returning almost to its original condition.

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We wish to thank Dr Bernhard Katz and Mr A. L. Hodgkin for reading the manuscript.

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## THE COMPOSITION OF ALVEOLAR AIR

BY I. F. S. MACKAY

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IN 1905 Haldane & Priestley pointed out that in order to obtain a satisfactory sample of alveolar air by their method the dead space of the lungs must first be washed out by the lung gases in the delivery of the sample. If more than this volume is expired the composition of the

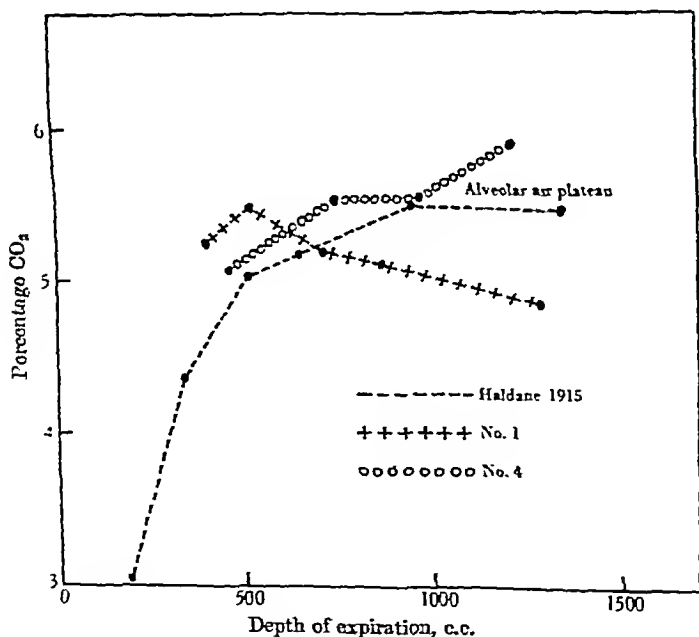


Fig. 1.

sample is the same whatever the depth of the expiration. In 1915 Haldane extended these investigations and obtained similar results. His results when set out graphically produce a plateau which represents the constant partial pressure of  $\text{CO}_2$  that is obtained when the dead space has been washed out (see Fig. 1).



The presence of this "alveolar air plateau" has been demonstrated in only one subject (J. S. H.). Partly on this account and also because of other work that the writer has carried out, it was doubtful if it was always present. It was therefore decided to investigate this problem in a small number of subjects.

### METHODS

The first steps taken in these investigations were to reduce the experimental variations which are met with in the original Haldane-Priestley method.

Instead of sealing the end of the tube by means of the tongue, and then withdrawing a sample into a sampling tube, two taps were used to form a trap. These were placed as close as possible to the mouthpiece (in fact, the first tap acted as the mouthpiece) so as not to increase the dead space of the apparatus any more than was necessary; also for the same reason the volume of the trap between the taps was approximately 60 c.c. The samples were taken directly into a small Haldane gas analysis apparatus. A Hutchinson spirometer was used for measuring the volume expired, and was attached to the end of the Haldane-Priestley tube. When this read the desired volume the two taps were turned synchronously and as rapidly as possible.

The writer is satisfied that a sample which is trapped in this manner when the spirometer reads 1000 c.c. is the sample that issued from the mouth after 1000 c.c. of lung gases had been expired. This cannot be claimed by other methods except perhaps by the original Haldane-Priestley method.

In justification of this procedure a series of results is given in Table I of the percentage of CO<sub>2</sub> in end-expiration samples delivered by the original Haldane-Priestley method and by the modified method just described. The range of each series is shown with the average of the determinations. It can be clearly seen that the variations of the modified method are less.

TABLE I

No. of series	CO <sub>2</sub> percentage in end-expiration samples of alveolar air collected by the Haldane-Priestley method		CO <sub>2</sub> percentage in end-expiration samples of alveolar air collected by the modified method at the same sitting	
	Range	Average	Range	Average
1	5.39-5.91	5.57	5.44-5.49	5.47
2	5.12-5.69	5.42	5.25-5.35	5.29
3	5.39-5.52	5.46	5.40-5.47	5.44
4	5.03-5.76	5.43	5.51-5.57	5.55
	Final average	5.47		5.44

All results are expressed as a percentage of the prevailing atmosphere.

Throughout the following experiments where it states that alveolar air samples were taken it means that end-expiration samples were obtained by the modified method just described.

It has been shown previously [Mackay, 1939] that the level of the venous  $\text{CO}_2$  tends to settle down during an experiment while the subject is resting and it has been found that a similar fall occurs with the alveolar  $\text{CO}_2$ . For this reason a period of rest was observed before each experiment. To avoid the production of false results due to this falling of the alveolar  $\text{CO}_2$  the following procedure was observed. Firstly, the volumes were sampled in the order from the deepest to the shallowest. Secondly, they were taken in the reverse order. Finally, the samples were taken at random. The average time for an experiment of 5-6 determinations was 30 min. It was found wise to consider the first two sets of determinations as trial experiments, as most of the subjects showed an irregularity at the commencement of a series which was probably due to their inability to deliver consistently correct samples. In this series emphasis has been laid on the subject delivering each sample in a constant manner.

Another important point is the time taken to deliver a sample. If a subject was unduly long in delivering samples a curve showing an upward trend instead of a plateau might be obtained. All the subjects studied were so instructed that the time taken to deliver their largest sample was considerably shorter than the time taken for an ordinary expiration. In Table II are given the averages from the results obtained from the series of experiments that were performed on each subject.

TABLE II

	Subject no. 1, 8 experiments			
Vol. of expiration, c.c.	0-400	400-800	800-1200	1200-1600
$\text{CO}_2$ %	5.17	5.59	5.61	5.20
	Subject no. 2, 6 experiments			
Vol. of expiration, c.c.	0-400	400-800	800-1200	1200-1600
$\text{CO}_2$ %	5.79	6.24	6.18	6.18
	Subject no. 3, 4 experiments			
Vol. of expiration, c.c.	400-600	600-800	800-1000	1000-1200
$\text{CO}_2$ %	4.70	5.05	5.12	5.23
	Subject no. 4, 6 experiments			
Vol. of expiration, c.c.	0-350	350-700	700-1050	1050-1400
$\text{CO}_2$ %	4.58	5.08	5.42	5.56
	Subject no. 5, 6 experiments			
Vol. of expiration, c.c.	0-400	400-800	800-1200	1200-1600
$\text{CO}_2$ %	5.63	5.79	5.82	6.09
	Subject no. 6, 3 experiments			
Vol. of expiration, c.c.	200-600	600-1000	1000-1400	1400-1800
$\text{CO}_2$ %	4.72	4.58	5.01	5.36

Examination of the results in Table II shows that the results fall roughly into two groups: (a) Those that show a continuous rise with the volume of air that leaves the lungs. This was found in subjects 3, 4, 5 and 6 (see Fig. 1). (b) Those that show an initial rise, which is followed by a fall. This was found in subjects 1 and 2. Only in one out of 33 determinations on the six subjects was a plateau observed.

As these results vary so markedly from those of Haldane and Haldane & Priestley, a careful examination of their methods and results is necessary before conclusions and comparisons may be drawn.

Haldane & Priestley in their original experiment in 1905 state that the subject's tidal air was 600 c.c. and that the samples were collected at the end of inspiration. They were therefore able to obtain samples of alveolar air by the expiration of a volume less than the tidal air, namely, 492 c.c.

In 1915 Haldane repeated the experiment but altered the procedure. Instead of expiring a certain quantity and then sealing the mouthpiece with his tongue, he attached a rubber bag to the distal end of the tube, the capacity of which when inflated could be varied at will by the adjustment of a large wooden clamp. It was demonstrated on this second occasion, from the averages of the results obtained, that 950 c.c. were required in order to reach the "alveolar air plateau" (see Fig. 1). He does not state whether the samples were delivered at the end of inspiration or at the end of expiration. Later, in his book on *Respiration* [1922], he says that the figures were obtained from end-inspiration samples. In a second edition in 1935 he states that it was at the end of expiration that the samples were delivered. Assuming that this last statement is correct, it means that from the end-inspiration point 1550 c.c. of lung gases had to be expired to give a sample of alveolar air in 1915, while only 492 c.c. were necessary in 1905—a difference of over a litre! It is difficult to understand how such a variation in results was produced unless it was due to the difference in the apparatus.

Aitken & Clark-Kennedy [1928] devised an apparatus to withdraw a series of samples from one expiration. Their observations were made during muscular exercise for the purpose of determining the variations in the alveolar air during the respiratory cycle. They state that "if the variation in the composition of the air *passing out of the mouth* [their italics] in a single expiration is observed it is possible to deduce what changes must have occurred in the alveolar air to give rise to the observed changes in the expired air". They do not state on what grounds this statement is true.

Following inspiration, the gases in the lungs must tend to go into equilibrium with the gases in the mixed venous blood entering the lungs. This is what occurs when the breath is held, but normally the gases are swept out in the expiratory phase before this stage is reached. If samples were taken *inside the lungs* by means of a catheter this should be demonstrated. The writer has shown that samples taken from air *passing out of the mouth* do not necessarily conform to these changes that occur in the lungs.

In their complicated apparatus there must have been considerable mixing of the lung gases as they issued from the mouth. The trapping of the samples was not carried out in a manner comparable to that used by the writer, and they could not safely assume that a sample obtained at say 1000 c.c. was the same sample that issued from the mouth at 1000 c.c. As their experiments were performed on a subject who had been exercising for 10 min. this may have produced results that varied from the resting state. The results that they obtained were on two subjects, and showed a curve which rose from zero with an S-shaped inflexion and continued into a straight line with a gradual upward slope.

Pearce [1917] determined the alveolar  $\text{CO}_2$  from a formula. He publishes the results of two experiments which are similar to those of subjects 1 and 2. He states, "The percentage of  $\text{CO}_2$  is plotted on cross-section paper on the ordinates, with the cubic centimeter in the expiration on the abscissae. A line joining these should make a hyperbolic curve. Any marked deviation from such a curve indicates at once that an error has been made in taking the sample—and this sample should be discarded."

It is reasonable to assume that different parts of the lungs are ventilated in a different manner, so that when a forced expiration is made, air from those less well ventilated or "deeper" parts is exhaled. This might explain the type of result that is produced by subjects 3, 4, 5 and 6; but it is difficult to apply this argument to the results which were produced by subjects 1 and 2, unless the gases from the "deeper" or less well ventilated parts of the lungs are *shunted out* by a sudden forced expiration in front of samples from portions which are better ventilated.

#### SUMMARY

It is generally assumed since the work of Haldane & Priestley [1905] and Haldane [1915] that when once the dead space is washed out by the lung gases in the delivery of an alveolar sample the partial pressures of

the gases remain at a constant figure whatever the depth of the expiration. The writer has been unable to confirm the presence of this "alveolar air plateau" in any one of the six subjects investigated.

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## EFFECT OF GRAVITY ON THE BLOOD PRESSURE OF THE CAT

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*(Received 16 October 1939)*

THE effects of posture on the circulation, with full references to the literature, are described by McDowall [1938]. The present investigation has been carried out primarily to ascertain the role of the pressor receptor nerves in the compensating mechanism of postural change. An increased fall of blood pressure in the feet-down position after denervation of the carotid sinuses has been found in the dog by several workers [Hering, 1927; Bennati & Mazzucco, 1935; Wald, Guernsey & Scott, 1937]. J. Koch [1935], using rabbits, found a slight increase in the fall of blood pressure after denervation, and also that the compensatory mechanism is more easily exhausted under these conditions. In man, Mark & Neumann [1932] concluded that the sinus plays an important part in the regulation of the circulatory changes during an alteration of posture. McWilliam [1933] found a correlation between the effects of carotid sinus pressure and the increase in pulse rate on sitting up, but not in the further change from sitting to standing.

Preliminary experiments appeared to indicate that in the cat, the role of the pressor receptor nerves in the control of the circulation during changes of posture, was not so important as had been concluded from the above work [Edholm & McDowall, 1936]. It therefore seemed expedient to reinvestigate the whole problem.

In the cat, as in man, the effects of putting the animal in the feet-down position are an immediate fall in the carotid blood pressure followed by a variable rise. This marked variation introduced a difficulty in that one could not draw conclusions by comparison without risk of error. An attempt has been made to establish a standard for cats as a whole by examining a large series and taking average results.

This paper describes the effect of gravity on the blood pressure of fifty-six cats, together with the results obtained from a further sixty-four

cats on which various experiments were carried out. The results have been treated in a way that enables them to be used as a standard.

The results show that:

- (1) Compensation in the cat is more effective than earlier workers had found.
- (2) It is not so good as in man.
- (3) It is related to the initial blood pressure.
- (4) It becomes progressively less in the course of a given experiment.
- (5) It is related to the sex and weight of the animal.
- (6) Removal of the carotid sinus receptors does not affect the response.
- (7) Removal of the aortic pressor receptors diminishes powers of compensation.

#### METHODS

Cats anaesthetized with ether followed by chloralose, were used for all experiments (chloralose, 0.056 g. per kg. body weight). Experiments were not begun until half an hour after the ether had been given, by which time most of the ether had been excreted.

The table used consisted of a flat board on which the animal was firmly fixed, rotating about a transverse horizontal axis. The table was constructed so that the axis of rotation passed 2 in. above the level of the board. The animals were fastened on the board with the axis of rotation passing about 5 cm. caudally to the right auricle. All animals were rotated about the same point, to have comparable results. According to Clark, Hooker & Weed [1934] rotation about this point reduces the hydrostatic effects to a minimum.

Blood pressure was recorded continuously on a kymograph by a mercury manometer connected to a cannula in the central end of the right common carotid artery. A tracheal cannula was inserted, and both vagi and the left carotid artery exposed before experiments began. Since the right carotid artery was necessarily ligatured for the recording of blood pressure, the right carotid sinus was in effect out of action, but in addition this sinus was always denervated.

Sudden movements were avoided when tilting the animal. About 2 sec. was taken to move the table from the horizontal to the vertical, 4 sec. being the maximum time ever used. Within these limits, no appreciable alteration of the blood-pressure curve was observed, but if a longer time is employed, the compensatory mechanism begins to act before the animal is in the vertical position, and the full fall of blood pressure may not be seen.

Animals were maintained in the vertical position for 2-5 min. and then returned to the horizontal position. Experiments carried out for longer periods showed that the blood pressure reached a steady state in the large majority of animals within 2 min.

Since the animals were not rotated about the point of the carotid cannula, an error is introduced into the kymograph record. The distance from the tip of the cannula varied slightly from cat to cat, but averaged 12 cm. so when the animal was in the vertical position, the blood pressure as recorded was in all cases approximately 1 cm. Hg too high. This error is present in all experiments and, except when plotting Fig. 4, has not been otherwise considered.

To test the effect of the carotid sinus, a clip was placed on the left common carotid artery. This procedure is equivalent to temporary denervation, as Bronk & Stella [1932] have shown that reduction of the endosinusal pressure to 50 mm. Hg or lower abolishes the discharges from the end-organ, and von Euler & Liljestrand [1936] find that clipping the common carotid lowers the endosinusal pressure below this level, the fall persisting until the clip is removed.

## RESULTS

The first experiments described were carried out on fifty-six cats, and a further sixty-four cats have been studied under initially different conditions.

### *The normal response*

Tilting an animal into the feet-down position, produces a variable effect on blood pressure. (The feet-down position means the rotation of the animal into the vertical with the head up and the hindlegs down, and will be referred to as the F.D. position.) In Fig. 1 are shown the blood-pressure tracings from four different experiments, illustrating this variability of the blood-pressure record.

The results have been classified as follows:

(a) Rise of blood pressure above the original level, which is maintained, the average blood pressure in the F.D. position being higher than in the horizontal. This has been obtained in five experiments.

(b) Fall in blood pressure followed by a rise above the original level, but with the average blood pressure remaining below the horizontal level. Obtained in six experiments.

(c) In the majority of cases, placing an animal in the F.D. position produces a fall of pressure, followed by a varying degree of recovery;



this recovery not being adequate to bring the blood pressure back to original level. The greatest fall of pressure is in the first 30 sec. Obtained in thirty experiments.

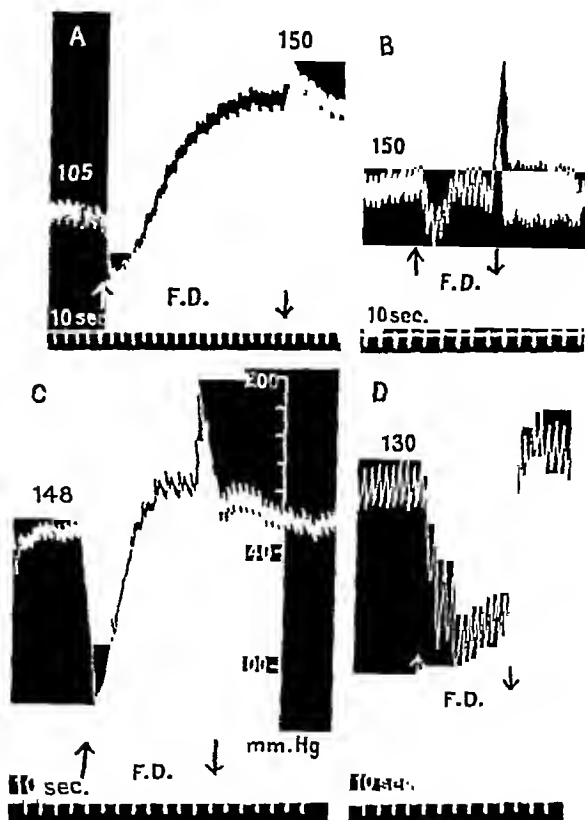


Fig. 1. Blood-pressure tracings obtained on tilting the animal into the F.D. position followed by return to horizontal.  $\uparrow$  marks movement from horizontal to vertical.  $\downarrow$  marks return to horizontal. Results obtained in four different animals on the first tilt, showing the wide variation of the blood-pressure fall and degree of recovery.

(d) The blood pressure falls sharply and shows no recovery, or may continue to fall more or less sharply for the whole period of the experiment. This occurred in fifteen experiments. The difference between "c" and "d" is that in "c" the initial fall is greater than the final fall, and in "d" the reverse is true.

In view of the divergence of the results the following methods of analysis were used.

*The change in blood pressure*

(1) Measurements were made on the blood-pressure tracing, of the initial change, the final level just prior to replacing the animal in the horizontal position, and two or three points in between, depending on the irregularity of the curve. The final figure for each experiment has been calculated as the average change of pressure in the F.D. position, expressed as a percentage of the original level, i.e. the level of blood pressure in the horizontal position.

TABLE I. Initial and final falls of pressure in the F.D. position, expressed as percentage of lying pressure

Initial blood pressure	First F.D.		Subsequent F.D.	
	Initial fall	Final fall	Initial fall	Final fall
190	11.0 $\pm$ 2.0 (12)	8.7 $\pm$ 2.9	19.6 $\pm$ 2.9 (11)	11.8 $\pm$ 4.5
180				
170				
160	23.2 $\pm$ 3.0 (26)	12.5 $\pm$ 2.8	25.8 $\pm$ 2.0 (29)	17.9 $\pm$ 2.9
150				
140				
130	25.7 $\pm$ 4.4 (13)	19.4 $\pm$ 5.5	30.6 $\pm$ 2.9 (28)	28.3 $\pm$ 3.9
120				
110				
110	47.7 $\pm$ 8.5 (5)	29.3 $\pm$ 13.5	41.3 $\pm$ 2.3 (9)	34.1 $\pm$ 9.7
90				
80				
50				

TABLE II. Effect of weight. First F.D. only

Weight in kg.	Blood pressure	% fall	Mean fall	Initial fall	Final fall
5.0	150	4.3 $\pm$ 0.1 (1)	5.0 $\pm$ 0	7.3 $\pm$ 0	1.3 $\pm$ 0
4.0	175	12.9 $\pm$ 2.3 (2)	14.8 $\pm$ 3.0	11.5 $\pm$ 1.3	14.2 $\pm$ 4.5
3.5-4.0	166	16.9 $\pm$ 3.4 (11)	22.1 $\pm$ 4.4	18.2 $\pm$ 3.4	13.2 $\pm$ 3.7
3.0-3.5	165.2	18.1 $\pm$ 3.4 (27)	20.0 $\pm$ 3.7	21.8 $\pm$ 3.5	14.7 $\pm$ 2.6
2.5-3.0	158.5	16.3 $\pm$ 2.4 (35)	18.9 $\pm$ 2.9	20.8 $\pm$ 2.3	11.5 $\pm$ 2.4
2.0-2.5	152.3	20.6 $\pm$ 5.1 (16)	22.7 $\pm$ 4.5	21.8 $\pm$ 5.1	16.9 $\pm$ 5.2
1.5-2.0	141.0	10.8 $\pm$ 5.4 (6)	12.11 $\pm$ 6.6	17.1 $\pm$ 4.5	9.7 $\pm$ 8.2

(2) As a check on the first method, the blood-pressure curves were examined by measuring the area of the curve above and below a base-line, this line being the prolongation of the initial blood-pressure level. Areas above the base line were given a positive value, and those below a negative value, the final result being a sum of these two measurements. The figure obtained is converted into the mean blood-pressure change. The area is measured in terms of sq. mm. =  $A$ , and the length of each record measured in mm. Then  $A/\text{length}$  will represent the vertical distance in mm. on the tracing separating the initial blood-pressure level and the mean blood-pressure level in the F.D. position.

The percentage blood-pressure change and the mean blood-pressure change as measured above were estimated for each experiment. The results obtained by the two methods are essentially the same, so only the first method has been used in this paper, except in Table II. The standard error has been calculated for all averages and is indicated on the charts by short vertical lines.

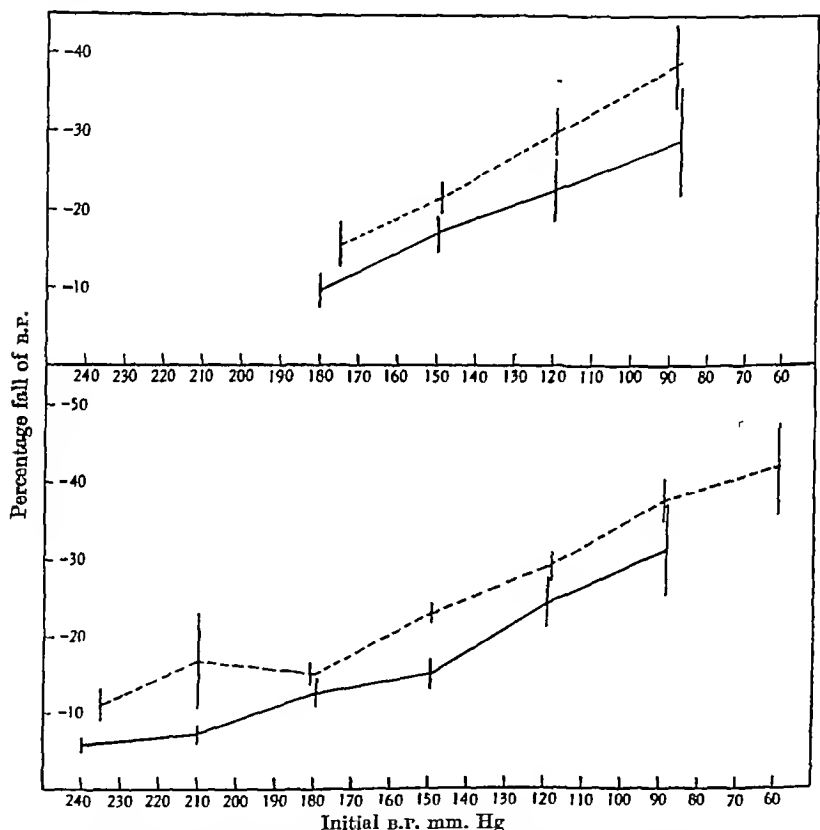


Fig. 2. Abscissae: initial level of blood pressure in lying position, measured just prior to tilting the animal. Ordinates: percentage fall of blood pressure in F.D. position. Top half: results obtained in animals with pressor receptors intact. Lower half: combined results from all experiments. — continuous line, results obtained at the first F.D. --- broken line, results obtained at all subsequent experiments.

#### *Relationship between initial blood pressure and change of pressure*

The results have been arranged in groups according to the initial level of blood pressure, and the average percentage change worked out. The results are shown in Fig. 2, upper half. In no group is the average

blood pressure raised above the original level. There is a clear relationship between the initial level of blood pressure and the average fall of pressure in the F.D. position; the fall, both absolute and percentage, becoming progressively greater with decrease in the initial level. These results are taken from the first experiment carried out on each animal; fatigue is not a factor.

### *Fatigue*

When a F.D. experiment is repeated several times on the one animal it is found that the compensatory mechanism gradually becomes less effective, so the fall of blood pressure in the F.D. position is greater with

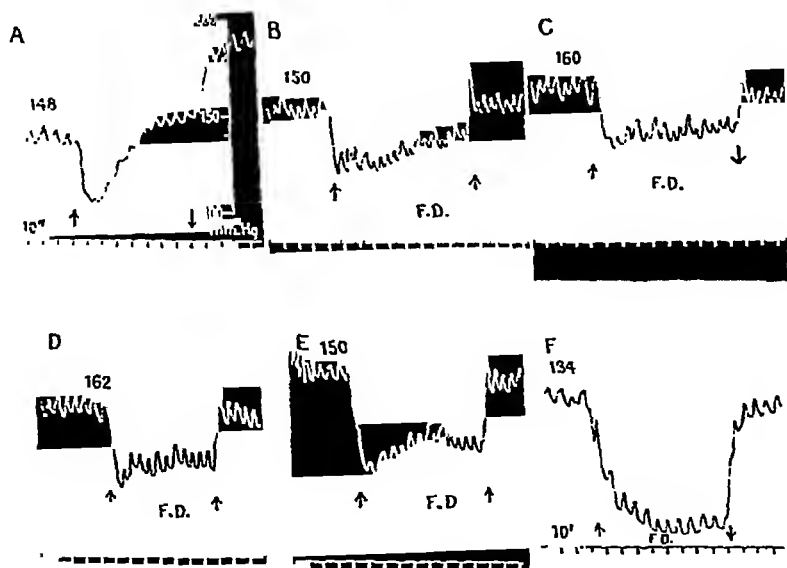


Fig. 3. The effect of repeated tilts. Tracings obtained in one animal on first and subsequent tilts. A, first F.D.; B, second F.D., etc. Point of movement marked by arrows. The fall of blood pressure in the F.D. position increases with each experiment, and the degree of recovery becomes less.

each experiment. At the same time there is usually a gradual fall of the general level of blood pressure, so that each successive experiment is performed at a lower initial level. Fig. 3 shows the changes in blood pressure produced by a series of F.D. experiments in a typical case. The interval between each tilt is 5-10 min., so that the blood pressure has an adequate time to reach a steady state.

In the upper curve of Fig. 2 the percentage blood pressure changes for all subsequent experiments, i.e. for all except first F.D., have been plotted. Two deductions can be made, (i) that the fall of blood pressure in the F.D. position shows the same relationship to the initial level as in the first F.D. experiments, (ii) that the fall of pressure is not an effect of the concomitant fall in general level but is a true effect of fatigue. (Occasionally it was found that the fall of pressure at the third F.D. might be less than at the first or second F.D., but the average results show a steady increase in the fall with successive F.D.)

### *Recovery phase*

As shown in Figs. 1 and 3, after the sharp initial fall of pressure in the F.D. position there is a period of recovery of varying degree. This recovery phase is an index of the compensatory powers of the circulation. The average initial and final falls in the F.D. position are given in Table I. The final fall is measured just prior to replacing the animal in the horizontal position, the difference between the initial and final fall measuring the degree of recovery. The degree of recovery is marked at all levels of blood pressure for first F.D. experiments, but is considerably less for subsequent experiments, as is seen in Fig. 3. There is a greater increase in the final fall than the initial fall in subsequent experiments, so that fatigue chiefly affects the degree of compensation.

The average recovery for first F.D. experiments is  $8.6\% \pm 2.0$  (standard error, fifty-six experiments), and for subsequent experiments  $5.7\% \pm 1.8$  (seventy-seven experiments). The results are expressed as a percentage of the blood pressure in the lying position, the average for first F.D. being 144 mm. Hg and for subsequent 135.5 mm. Hg.

### *Relationship between horizontal and vertical blood pressures*

Schneider & Truesdell [1922] measured the change in systolic blood pressure in healthy young male adults, 2 min. after standing up. Estimating the relationship between standing and lying figures they found that taking the lying figures as the basis, at all levels of blood pressure there was a rise of 2–3 mm. Hg in the standing position. Taking the standing values as their basis they found that the groups with a low standing pressure had a higher lying pressure, in the middle range the standing and lying pressures were almost identical, and the high-pressure standing group had a lower lying pressure.

In Fig. 4 the result of assessing the present experiment in a similar manner is shown; i.e. the change in blood pressure 2 min. after placing

the animal in the F.D. position has been compared with the initial level of pressure and vice versa. In this case so as to have as close an approximation as possible to Schneider's figures, 10.0 mm. Hg has been sub-

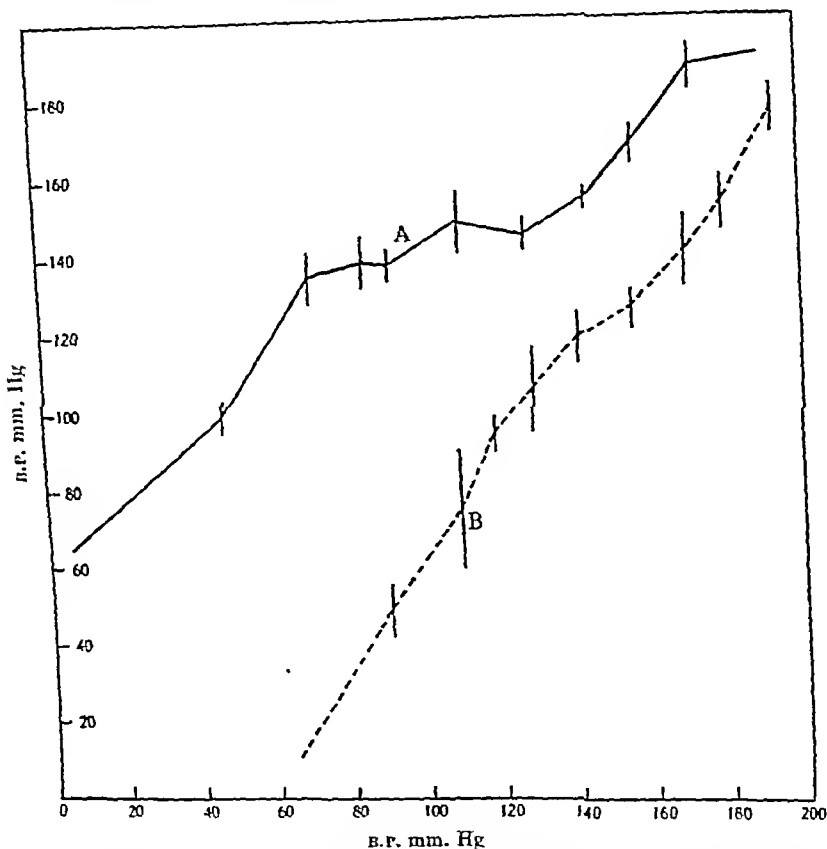


Fig. 4. The relation of blood pressure in F.D. position (standing), to pressure in the lying (horizontal) position, and vice versa. Curve A, continuous line: abscissae, blood pressure in F.D. position; ordinates, blood pressure in lying position. Curve B, broken line: abscissae, blood pressure in lying position; ordinates, blood pressure in F.D. position. 10 mm. Hg deducted from all F.D. blood pressures, to correct for movement of cannula. Blood-pressure level estimated after 2 min. in F.D. position. Only first F.D. experiments included.

tracted from all standing figures, this being the error introduced by the movement of the carotid cannula. Taking the lying value as a basis, it is found that at all levels of pressure the average (standing or) vertical pressures are always lower, and the difference between the two values increases with decrease in the lying pressure (absolute falls, not percentages

have been used). When the standing figures are used as the basis, the results are more similar to those of Schneider & Truesdell. At low standing pressures, the lying pressure is much higher, this difference decreasing with increase in the standing pressure, until at the highest range of pressures the two values are identical.

### *The effect of the pressor receptor nerves*

*General.* Experiments were first carried out consecutively; the first F.D. without the application of the carotid clip, the second F.D. with the clip applied, and the third with the clip removed. If the results had been clear-cut, i.e. if in all experiments when the carotid clip was applied, the fall of blood pressure in the F.D. position had been greater or less than the fall produced before and after the application of the clip, it would have been easy to draw a definite conclusion as to the role of the carotid sinus. The results have not been so conclusive. The fall of pressure in the F.D. position when the clip was applied was sometimes greater, sometimes less and occasionally identical with the previous fall.

To estimate the importance of the pressor receptor mechanism, all results obtained under similar conditions have been grouped together, and compared with results obtained under other conditions. There are four groups of experiments:

- (1) Normals: all pressor nerves intact (except right sinus).
- (2) Carotid clip applied: both sinuses out of action.
- (3) Both vagi cut: the two aortic nerves are divided.
- (4) Both vagi cut and the carotid clip applied; so removing the four main pressor receptors.

These four groups do not represent four separate batches of animals as experiments might be carried out on any one animal, which would come in all four groups. The sequence of experiments might be:

First F.D. with carotid clip applied.

Second F.D. without carotid clip.

Third F.D. following section of the vagi.

Fourth F.D. with carotid clip.

Fifth F.D. without carotid clip.

The first F.D. would be classified in group 2, the second in group 1, third in group 3, fourth in group 4, and the fifth in group 3.

*Results.* The average percentage blood-pressure fall in each blood pressure group for all the four different types of experiment are given in Fig. 5. All experiments performed under the same heading have been grouped together, i.e. both first and subsequent F.D., as the comparatively

small number of experiments in the first F.D. only give scattered results. The results in the first three groups are closely similar, but the average fall of pressure when the vagi are cut and the carotid clip applied is greater over a large section of the graph, although statistically the difference is not significant. The average falls measured in mm. Hg are as follows: normals,  $31.4 \pm 1.67$  (133); vagi cut,  $32.5 \pm 1.95$  (109); carotid clip,  $31.6 \pm 2.3$  (69); vagi cut and carotid clip,  $35.8 \pm 2.7$  (100). The figures in brackets are the number of experiments.

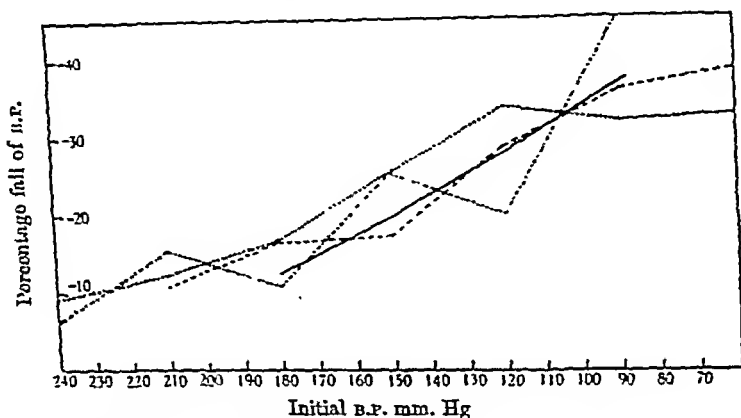


Fig. 5. Results obtained in normal animals, compared with those with one or more pressor receptors removed. Abscissae: initial lying blood pressure. Ordinates: percentage fall of blood pressure in the F.D. position. — normal animals; --- animals with vagi cut; ..... animals with carotid clip applied; - . - . animals with vagi cut and carotid clip applied. All experiments in each category combined, i.e. including first and subsequent F.D. experiments.

The initial and final falls of pressure of the four groups are shown in Fig. 6. The greatest recovery is seen in the carotid clip group, whereas the very slight recovery is seen in the two groups with the vagi cut. The average recovery in mm. Hg is: normals,  $9.9 \pm 1.45$ ; vagi cut,  $4.0 \pm 1.8$ ; carotid clip,  $15.8 \pm 1.5$ ; vagi cut and carotid clip,  $3.5 \pm 1.35$ .

The average initial fall is almost identical for all the groups.

#### *Effect of initial blood pressure, etc.*

In the first part of this paper the various factors influencing the response of the circulation to the F.D. position were discussed, and the results of experiments on fifty-six animals were described. In assessing the influence of the pressor receptor nerves, a further sixty-four animals were used, and the percentage blood-pressure fall, and initial and final



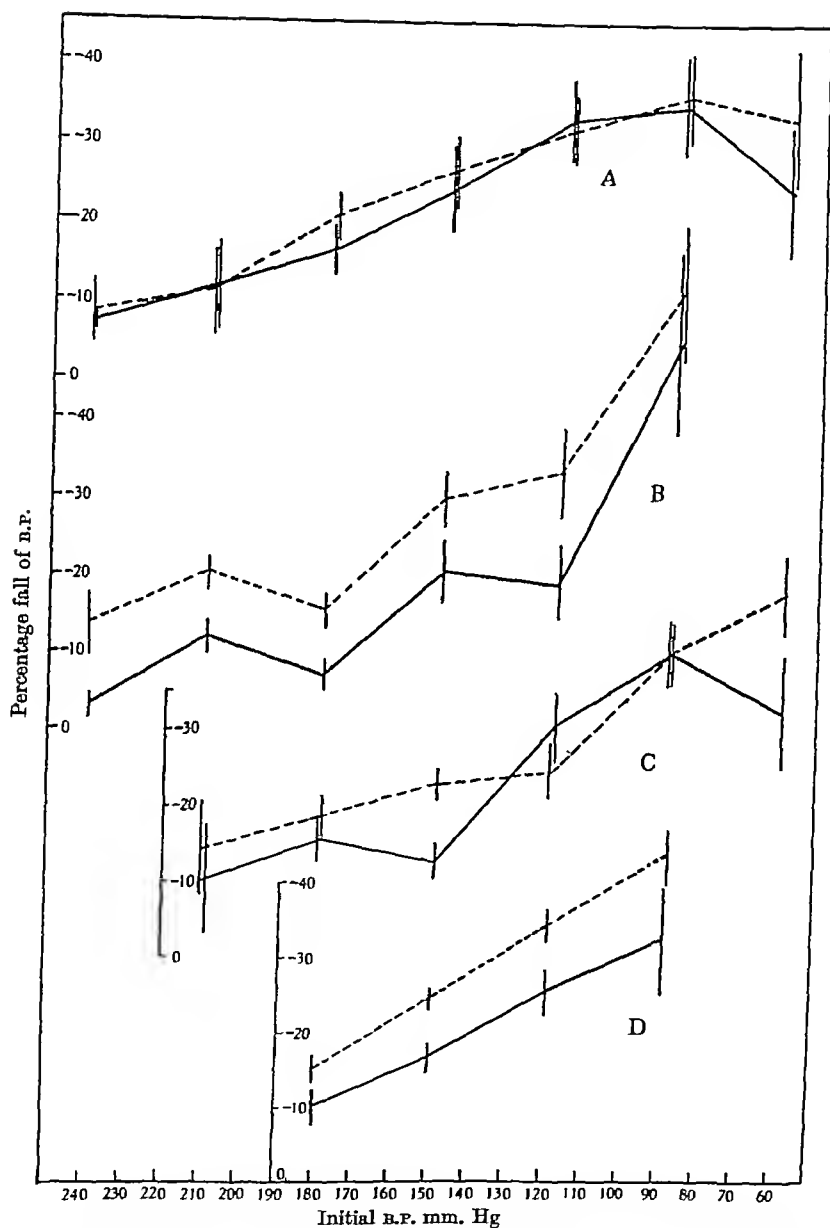


Fig. 6. Initial and final falls of pressure in normal animals and those with pressor receptors removed. A, animals with vagi cut and carotid clip applied. B, animals with carotid clip applied. C, animals with vagi cut. D, animals with pressor receptors intact. — indicates final fall of pressure; --- indicates initial fall of pressure.

falls in first and successive experiments have been calculated. The results are an amplification of those already given, i.e. there is a gradual increase

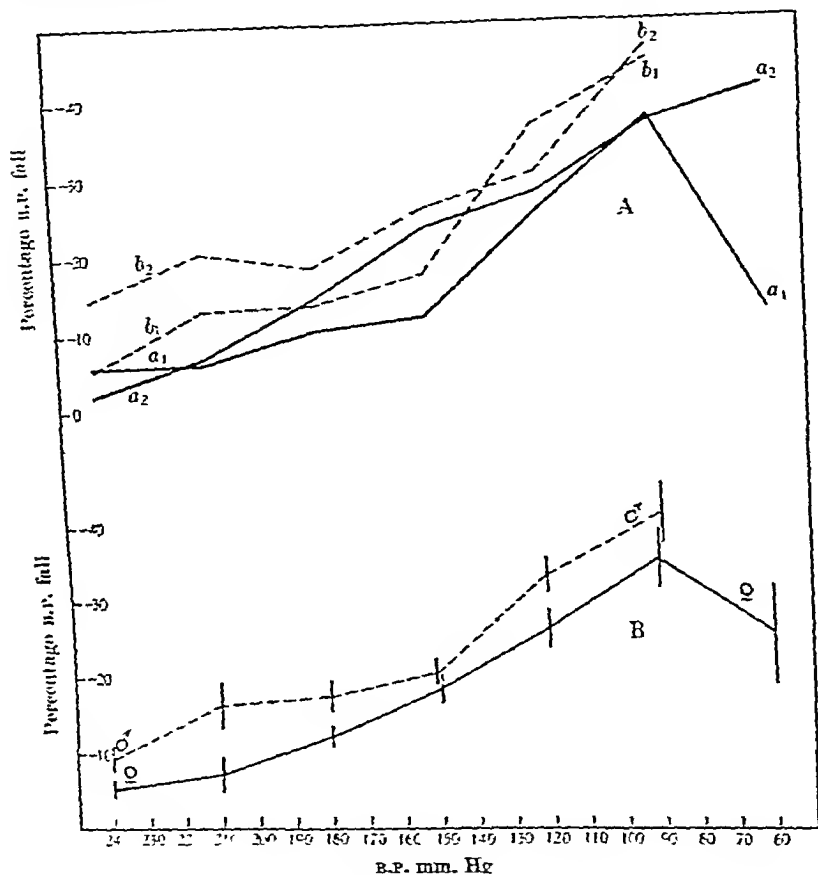


Fig. 7. Results obtained in males and females compared. All experiments combined, i.e. both first and subsequent experiments. — continuous line, results obtained in females. --- broken line, results obtained in males. Top curves show final and initial falls in males and females.  $a_1$ , final fall of pressure, females.  $a_2$ , initial fall of pressure, females.  $b_1$ , final fall of pressure, males.  $b_2$ , initial fall of pressure, males. Lower half of figure shows mean percentage fall of blood pressure in F.D. position in males and females.

in the fall of pressure in the F.D. position with diminishing initial pressure; the fall in successive F.D. experiments is greater than in the first experiments and the recovery is less.

In the lower half of Fig. 2 the blood-pressure falls for first and successive experiments are shown. The average falls in mm. Hg are for first

experiments  $26.0 \pm 2.0$  (119 exps.), and for successive experiments  $36.5 \pm 1.7$  (292 exps.); the average recoveries in the same order being  $12.25 \pm 1.4$ , and  $5.7 \pm 1.0$ .

#### *Influence of sex*

This factor was not discussed under the normal response as there were insufficient data. In Fig. 7 all results in males and females have been plotted. The average blood-pressure change in females is less than males at all levels of blood pressure, and both initial and final falls are less, with a greater degree of recovery in females. For the whole series of experiments these differences are not marked, as the average blood pressure in males is higher than females. If the figures for the first three F.D. experiments only are taken, the average blood pressures are almost identical, 160 mm. Hg in the male, 159.8 mm. Hg in the female. Initial falls are similar, but the final fall is greater in the male; 24.2 and 23.7 % being the initial fall in male and female respectively, 20.1 and 15.7 % being the final fall. The degree of recovery in the male is  $4.1 \% \pm 1.12$  (105 exps.) compared with  $7.9 \% \pm 1.5$  (113 exps.) in the female. This difference is statistically significant.

#### *Weight*

The effect of weight on the response is shown in Table II. All first F.D. experiments are included. The variation from group to group is small.

#### DISCUSSION

It is interesting that quite a large number of animals did not show a fall in blood pressure when placed in the F.D. position. L. Hill [1895] gives one tracing of the carotid blood pressure in a monkey which shows a slight rise of blood pressure, but a fall of pressure was apparently obtained in most monkeys. In other animals (cat, dog, rabbit), published tracings of various authors invariably show a fall of pressure in the F.D. position.

Most experimental results show a considerable difference between man and animals in the degree of compensation in the upright position, the blood pressure in man even being slightly higher on standing than lying (Schneider & Truesdell, 1922). This difference has been assumed to be due to the use of anaesthetized animals, and the normal erect posture of man. Loman, Damashek, Myerson & Goldman [1936a, b] made direct estimations of intra-arterial blood pressure in the carotid artery in man, taking a continuous record. They found a steep initial fall of pressure on tilting into the F.D. position, averaging 33 mm. Hg,

followed by a degree of recovery, the average final value being 21 mm. Hg. These figures, obtained in unanaesthetized men, are very similar to those obtained in the present research, using anaesthetized cats. In both cases the mean pressure is recorded, not the systolic. The initial fall of pressure would not be observed measuring the blood pressure 2 min. after tilting, as was done by Schneider & Truesdell.

The relationship between the initial blood pressure and the fall of pressure in the F.D. position has not been previously shown in animals. Loman *et al.* [1936a] observed in a high blood-pressure group suffering from arteriosclerosis, a marked fall of blood pressure on standing. Roth [1936] found that the standing systolic pressure in several cases of hypotension was higher than the reclining.

It would seem that cats with a high blood pressure have a strong arteriolar tone, so that there is only a slight relaxation in the vertical position, whereas in animals with a low blood pressure and a poor vascular tone, the vessels dilate under the extra strain of the vertical position.

The effect of fatigue and illness on the fall of pressure in man has been observed [Burger, 1925; Turner, 1929; Schneider & Truesdell, 1923; Hill, 1895] and J. Koch [1935] using rabbits observed that the compensatory mechanism becomes exhausted with repetitive experiments. This gradual failure of compensation is of great importance in experimental work, as results obtained in the same animal are not strictly comparable. It is misleading to compare results obtained before and after some experimental procedure, unless this gradual failure is taken into account.

Under chloralose anaesthesia, there is a gradual fall of blood pressure commencing about 2 hr. after the beginning of the experiment. There may be a gradual progressive vaso-dilatation, which is checked by compensatory mechanisms so the fall of pressure in the F.D. position is increased as the compensatory mechanism cannot exert its usual effect as it is already in play. If this is correct, experiments should be compared with each other on the basis of the time interval elapsing between the commencement of the anaesthesia, and the tilting experiment. In this research, the first F.D. was performed approximately  $\frac{1}{2}$  hr. after the animal was fully anaesthetized, and succeeding experiments were carried out at intervals of 10 min., so the time factor has not been separately considered.

No reference to sex differences in animals has been found in the literature. In man, Roth's results [1936] show a greater increase in pulse rate in males than females on standing. Proger, Dexter & Bock [1934]

consider that there is no difference in the postural response, but according to their published figures, there is a greater increase in pulse rate on standing in males.

The weight of the animal does not appear to be of great importance. In the range from 2.0 to 3.5 kg. the differences between the groups is very slight. In the lightest group, 1.5–2.0 kg. (6 % of animals) the small fall of blood pressure may be explained by the fact that these animals are probably young and active, and possibly have a more efficient vasomotor control. However, Schneider & Crampton [1936], studying the effect of posture on boys, found that they showed greater changes in pulse rate and greater falls of blood pressure than did adults.

*The role of the carotid sinus and the aortic depressor nerves*

The results obtained in this research differ in many respects from those obtained by other workers. The following reasons are suggested to explain these differences.

(1) The animals used. Hering [1927], Bennati & Mazzucco [1935], Wald *et al.* [1937], all used dogs, and J. Koch [1935] used rabbits. It is reasonable to suppose that these animals differ from the cat, especially as published tracings show that the recovery of blood pressure in the F.D. position in these animals is small, both before and after denervation of the carotid sinuses.

(2) The anaesthetic used is important as first emphasized by L. Hill [1895]. Wald *et al.* used morphia which depresses the respiratory centre and so may interfere with the respiratory pump in the F.D. position.

(3) The most serious criticism of previous work is that the basis of comparison is not sound. The effect on the blood pressure of tilting animals into the F.D. position before and after denervation of the carotid sinuses and section of the vagi, was the method employed in every case. The fall of pressure after denervation being greater than before it was concluded that the pressor receptors played an important role in preventing the fall. It has been shown in the first part of this paper that repeated tilts produce an increasing blood-pressure fall, so that even if the sinuses had not been denervated, a greater fall of blood pressure would have been observed. In some cases the difference before and after denervation have been greater than can be accounted for on the grounds of fatigue, but the results can only be considered suggestive and not conclusive.

In this paper it has been shown that removal of the carotid sinus impulses does not impair the vascular response to tilting. In fact the

average recovery is slightly greater than in the normal animal. After section of the vagi, the degree of recovery is significantly less than in the controls, although the initial fall is almost identical. Removal of the carotid sinus impulses with the vagi cut diminishes the recovery slightly but not significantly.

The average initial fall is similar in all four groups, so this is not affected by removal of the pressor receptors. It would appear that the aortic depressors are more important in compensating for the effects of gravity than the carotid sinus. Since the only practical method of dividing all the aortic depressor fibres is to cut the main vagus trunks, it is possible that other mechanisms involved in the vascular reaction to gravity have been abolished by removing the afferent and efferent nerve paths carried in the vagus trunk.

It is concluded that in the cat, there are other and more important mechanisms than the pressor receptor nerves involved in the vascular response to gravity.

#### SUMMARY

1. The effects of gravity on the blood pressure have been studied in 120 cats.

2. There is considerable variation in the effect, either a rise of blood pressure, no change, or more commonly, a fall with different degrees of recovery in the F.D. position.

3. The fall of blood pressure increases with decrease in the initial blood pressure.

4. Repeated experiments on the same animal produce a greater fall of pressure even at the same initial level of blood pressure.

5. Removal of the carotid sinus impulses does not affect the response.

6. Section of the aortic depressor nerves decreases the degree of recovery.

7. There is an average greater fall of pressure with smaller recovery of blood pressure in males than females.

8. Weight does not affect the response, except that there is a smaller fall in the lightest animals.

I am greatly indebted to Prof. McDowall for his advice and help in carrying out this work; to Prof. H. Barcroft for his advice in the presentation of the results. The first part of this work was carried out as a Bovril Research scholar at King's College, London.

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# THE RENAL EXCRETION OF INULIN AND CREATININE BY THE ANAESTHETIZED DOG AND THE PUMP-LUNG-KIDNEY PREPARATION

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THE evidence in favour of regarding the inulin plasma clearance as an accurate measure of glomerular filtration rate in normal animals has recently been reviewed by Smith [1937]. The primary aim of the present enquiry was to determine whether or not this concept could be extended to include the dog's isolated kidney perfused with defibrinated blood from a pump-lung circulation. To decide this question we have compared the inulin and creatinine clearances, which are identical in the normal animal [Shannon, 1935, 1936; Van Slyke, Hiller & Miller, 1935; Richards, Westfall & Bott, 1936]. We have made this comparison under a variety of conditions in the isolated kidney, and for reasons which will be apparent have extended our observations to the dog anaesthetized with chloralose.

In the anaesthetized, as in the normal dog, simultaneously determined inulin and creatinine clearances were found to be substantially the same. They differed significantly, however, in the isolated kidney excreting a concentrated urine. Further experiments were performed to determine the characteristic conditions under which this discrepancy appears. In the course of these experiments we have found that the isolated kidney differs markedly from that of the normal or anaesthetized animal in the extent to which the rate of glomerular filtration affects the fraction of filtered water which is reabsorbed.

## METHODS

The pump-lung perfusion arrangements were those used by Whittaker & Winton [1933]. Before connecting the kidney, inulin and creatinine were added to the defibrinated blood in amounts sufficient to yield concentrations of 150-200 and 20 mg./100 c.c. plasma respectively, and these concentrations were kept as steady as possible by an infusion replacing the amounts of these substances as they were lost in the urine.



Whenever the conditions were changed, time was allowed for endogenous urine to be formed, at least 3 c.c., to ensure complete washing out of previously formed urine, and then urine samples were collected during two or three consecutive periods under the same conditions.

The observations on *anaesthetized dogs* were performed after induction with chloroform and ether followed by intravenous injection of 0.1 g./kg. chloralose. The left kidney was denervated and freed from all except vascular attachments, the walls of the vessels being stripped; the ureter was cannulated just beyond the pelvis. The right renal pedicle was ligated so as to reduce changes in the blood due to urine formation. An adjustable clamp was placed on the aorta just above or below the diaphragm, and the arterial pressure was recorded from the femoral artery. A concentration of 150–200 mg. of inulin and of 20 mg. creatinine per 100 c.c. plasma was first produced by intravenous injection and then held about constant by continuous infusion.

Blood samples were withdrawn at the time when the collection of the urine sample was begun, for owing to the dead space in the kidney when the rate of urine formation is low this procedure is more likely to yield plasma concentrations comparable with those of the urine sample than would collection of a mid-period sample of blood. Errors due to the timing of the blood sample were negligible as the plasma concentration was usually kept fairly steady and the periods were of short duration.

The *chemical methods* used for the determination of inulin and creatinine were described by Shannon [1935, 1936].

The blood samples were centrifuged immediately upon withdrawal, oxalate being added as the anticoagulant in experiments on anaesthetized dogs. The plasma was diluted as soon as centrifuged and its precipitation completed at the end of the experiment. The volume of each urine sample was read to 0.05 c.c. and the urine flow calculated from this value; the total amount was immediately diluted to 10 c.c. to prevent precipitation of the inulin, and later, on the basis of a preliminary determination of the creatinine U/P ratio, further diluted so that the creatinine concentration was the same as in the plasma; it was then precipitated with the same technique as was the plasma. All chemical determinations were done in duplicate and the mean used in the calculation of the clearance.

An extensive series of blank determinations was made. In the pump-lung-kidney preparation there was from 1.0 to 3.0 mg./100 c.c. "apparent inulin" in the plasma, while the urine contained negligible

The endogenous creatinine was usually about 1 mg./100 c.c.

determined at high creatinine plasma levels. The only correction applied to the data was to subtract 2.0 mg./100 c.c. from the plasma inulin values as analysed. This correction resulted in about 1% change in the inulin clearance since the plasma inulin was usually from 150 to 200 mg./100 c.c. In the anaesthetized preparation the plasma contained about the same amount of endogenous creatinine, but the inulin blank was considerably higher, due to the presence of the anaesthetic. In filtrates of urine diluted in proportion to the U/P ratio of inulin, the blank attributable to chloralose and its derivatives was 2-3 mg./100 c.c., the corresponding plasma filtrates yielding the same or slightly lower values. The total inulin blank was 3-5 mg./100 c.c. in the plasma and 2-4 mg./100 c.c. in the diluted urine. The concentration of endogenous creatinine in plasma ranged from 0.8 to 1.1 mg./100 c.c., the clearance being about the same as that of exogenous creatinine at the high plasma levels. We have applied no correction to the inulin or creatinine concentrations as analysed, as this correction would change the inulin clearance less than 1%, there being danger of over-correction, while the creatinine clearance would be unchanged.

### RESULTS

*The inulin clearance in the isolated kidney and in the anaesthetized animal and the influence of changes in arterial pressure on it.* A summary of the initial group of periods from the experiments on the pump-lung-kidney (blood of normal composition) and the anaesthetized animal is given in Table I. The absolute values of the inulin clearance in both

TABLE I. Summary of all experiments to show the magnitude of the inulin clearances during the initial group of observations in the pump-lung-kidney (P-L-K) and the chloralosed dog (C-D). The experiments starred are excluded from summaries of the data for reasons given in the text.

Exp. no.	Preparation	Animal weight kg.	No. of observations	Perfusion or blood pressure mm. Hg	Inulin clearance c.c./kg./min.
1 C	P-L-K	9.0	2	125	0.635
2 C	P-L-K	10.0	3	73	0.722
3 C	P-L-K	8.5	2	100	0.897
9 C*	P-L-K	9.5	2	109	0.121
10 C	P-L-K	7.5	3	66	0.757
11 C	P-L-K	9.5	3	165	0.625
12 C*	P-L-K	9.0	2	101	0.314
13 C	P-L-K	9.5	3	77	0.628
15 C	P-L-K	8.5	3	201	1.52
16 C	P-L-K	9.0	3	210	1.85
4 C	C-D	11.0	3	131	2.00
5 C	C-D	12.0	3	160	1.46
6 C	C-D	9.5	3	142	1.55
7 C	C-D	10.5	3	119	1.25
8 C	C-D	13.5	3	195	2.00

preparations are clearly well below those obtained in the normal animal. Most published work on the perfused kidney shows the same discrepancy. The average rate of glomerular filtration in normal dogs on a mixed diet is 94 c.c./min./sq. m. of body surface [Smith, 1937, p. 262]; i.e. for dogs of from 8 to 12 kg. of body weight, about 2.3 c.c./min./kidney/kg. The figures in the table show that clearances in the isolated kidney do not approach those in the normal dog even at high perfusion pressures. In the anaesthetized animal the clearances are midway between the normal animal and the isolated kidney, but this may well be due to uncontrollable variables such as operative trauma, blood loss, the rate of intravenous fluid administration, and the presence of anaesthetic in the animal, factors which may drastically affect the circulatory adjustment and, even in the absence of renal innervation, the rate of glomerular filtration.

The effect of change in arterial pressure on the inulin clearance is not very different in the two preparations, 0.7 c.c./min./kg./100 mm. Hg change in pressure in the anaesthetized animal, and 0.6 c.c./min./kg./100 mm. Hg in the isolated kidney. The difference in the two preparations can be attributed to the lower absolute clearances in the isolated organ. There is a quantitative difference in the effects of lowering and raising the arterial pressure in the isolated kidney, whereas in the anaesthetized animal the change in inulin clearance with change in pressure seems perfectly reversible. In all seven isolated kidneys subjected to both procedures, the change in clearance was relatively greater for a reduction than for an increase in pressure. Although this effect was larger in experiments in which the reduction preceded the increase, it was also present in three experiments in which the increase preceded the reduction. Lowering the pressure produced a mean of 0.75 c.c./min./kg./100 mm. Hg. Raising the pressure produced one of 0.45 c.c./min./kg./100 mm. Hg.

Two of the eleven isolated kidneys (9 C and 12 C, Table I) were regarded as too atypical to justify inclusion of the results obtained from them in the final analysis of the data. One was very tense *in situ*, about double the weight usual in a dog of its size, had two to three times the usual blood flow and showed an inulin clearance in its initial group of periods of only a small fraction of the usual value. The other kidney suffered from our employing a technique of excision substantially different from that usually employed; its inulin clearance during the initial group of periods was also very low.

*Creatinine/inulin clearance ratio*

No significant difference was found in the creatinine/inulin clearance ratios in observations on six chloralosed dogs. The details of a single experiment are given in Table II, and a summary of all the experimental

TABLE II. An experiment on a dog anaesthetized with chloralose showing the effect of changes in renal arterial blood pressure on the clearances of inulin and creatinine. The fraction of water reabsorbed is almost independent of the amount filtered. Exp. 7 C. Weight 10.5 kg. Kidney weight 35 g. 10.30 a.m., chloralose 100 mg./kg. after preliminary induction with ether and chloroform mixture. 11.00-11.50 a.m. abdomen opened, left kidney denervated and ureter cannulated, right renal pedicle tied off. Artificial respiration started and clamp attached to lower part of thoracic aorta. 11.50 a.m. inulin 2.5 g. and creatinine 1.0 g. intravenously in 15 c.c. of saline and an intravenous infusion of inulin 8.0% and creatinine 1.0% continued to end of experiment at the rate of 0.33 c.c./min.

Period	Time	Dura- tion min.	Vol. of sample c.c.	Plasma level		U/P ratio		Clearance		Clear- ance ratio Cr./In.	Blood pressure mm. Hg
				In. mg. %	Cr. mg. %	In.	Cr.	In. c.c./ min.	Cr. c.c./ min.		
Blood pressure existing in animal 132-100											
1	12.50	8	1.5	127	19.5	86.6	85.5	16.3	16.1	0.99	132
2		8	1.1	130	18.8	80.4	82.1	11.0	11.2	1.02	125
3		8	1.1	132	19.1	88.0	86.9	12.1	11.9	0.98	100
Spontaneous fall in blood pressure 80-83 mm. Hg											
4	1.15	15	0.6	139	19.4	155	150	6.20	6.0	0.97	80
5		15	1.2	150	18.7	103	109	8.64	8.72	1.01	83
Aortic clamp tightened 60-52 mm. Hg											
6	2.05	17	0.8	157	18.7	116	114	5.45	5.36	0.98	60
7		17	1.2	164	19.2	111	111	7.84	7.84	1.00	52
8		17	0.9	177	20.0	96.3	90.5	5.11	4.80	0.94	52
Aortic clamp removed 125-115 mm. Hg											
9	2.59	9	1.0	171	19.8	134.0	128	14.9	14.2	0.95	125
10		9	1.1	169	19.4	99.4	99.3	12.1	12.1	1.00	120
11		9	0.9	158	18.6	130.0	128	13.0	12.8	0.98	115

In. = inulin; Cr. = creatinine.

observations in Fig. 1. The mean creatinine/inulin clearance ratio of this series was 0.985 with a standard error of  $\pm 0.023$  (43 observations). This ratio is not significantly different from unity, and the creatinine and inulin clearances may be taken as identical in the anaesthetized dog as they are in the normal dog.

In the nine isolated kidneys, however, the simultaneously observed creatinine and inulin clearances were not always the same, as shown in Fig. 2. Up to a U/P ratio of about 40 there was a rough correspondence between the two, the mean ratio being  $1.005 \pm 0.08$  (64). At higher U/P ratios the creatinine clearance was systematically lower than the inulin

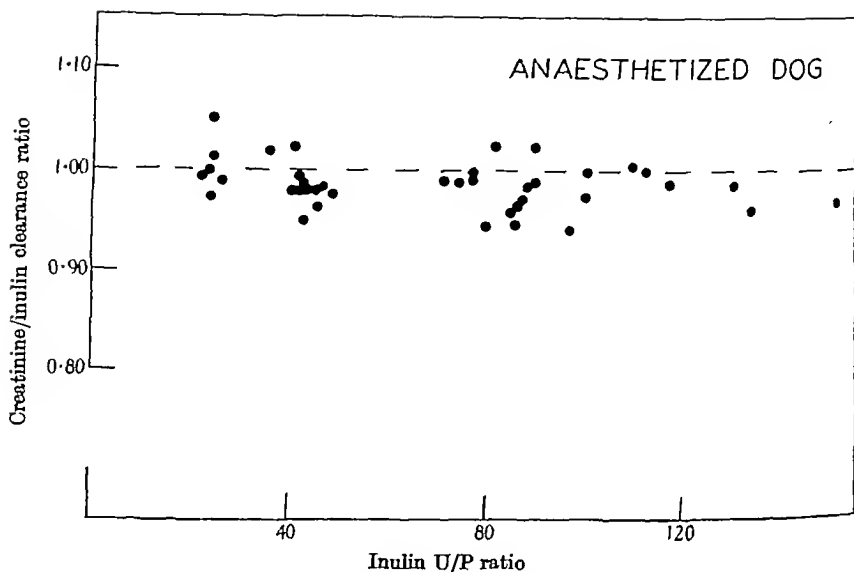


Fig. 1. Chart showing the relation between the creatinine/inulin clearance ratio and the U/P ratio of inulin in the kidney of the anaesthetized dog. Each point represents the ratio of creatinine clearance to the simultaneously determined inulin clearance in a single experimental period. The broken line represents a creatinine/inulin clearance ratio of 1.00.

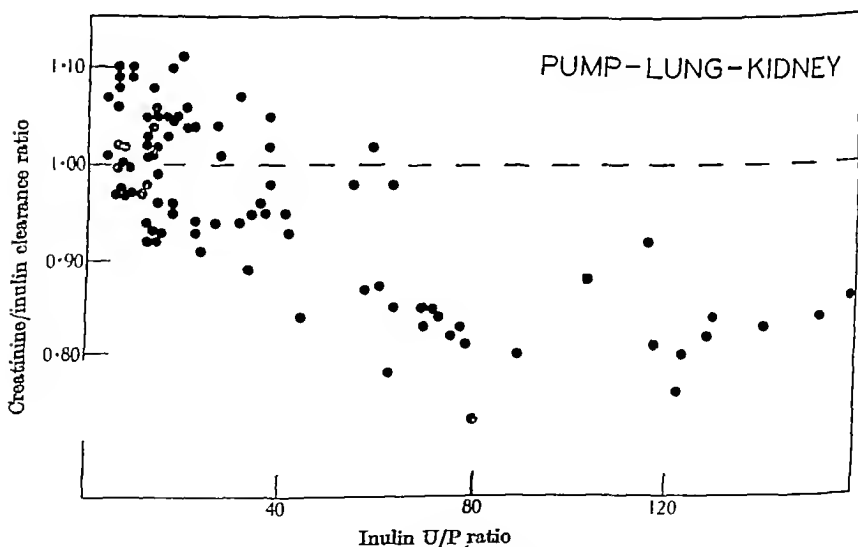


Fig. 2. Chart showing the relation between the creatinine/inulin clearance ratio and the U/P ratio of inulin in pump-lung-kidney preparations. Each point represents the ratio of creatinine clearance to the simultaneously determined inulin clearance in a single experimental period. The broken line represents a creatinine/inulin clearance ratio of 1.00.

clearance, the mean ratio of this group being  $0.86 \pm 0.06$  (28). Detailed results of our observations on one of the isolated kidneys are given in Table III, which shows the discrepant inulin/creatinine clearance ratio characteristic of the high inulin U/P ratios.

TABLE III. Pump-lung-kidney experiment (13 C) showing the effect of changes of perfusion pressure on the absolute clearances of inulin and creatinine, their ratio, and the extent of water reabsorption. Kidney weight, 40.5 g. Blood detoxicated by circulation through lungs 10.30 a.m. to 12.00 noon. Kidney attached 12.00 noon, first period started 1.10 p.m. Perfusion fluid 1 l. of defibrinated blood to which 1.8 g. of inulin and 0.3 g. of creatinine had been added. Throughout the experiment a solution of 4.0% inulin and 0.5% creatinine was added at the rate of 0.2 c.c./min. at low perfusion pressures and 0.4 c.c./min. at high pressures.

Period	Time	Dura- tion min.	Vol. of sample c.c.	Plasma level		U/P ratio		Clearance		Clear- ance ratio Cr./In.	Blood flow c.c./ min.	Inulin extrac- tion ratio
				In.	Cr.			In.	Cr.			
				mg. %	mg. %	c.c./ min.	c.c./ min.					
Perfusion pressure average 77 mm. Hg												
1	1.10	15	0.70	161	22.5	129	108	6.01	5.04	0.84	154	0.078
2		15	0.80	156	23.1	124	100	6.61	5.33	0.81	154	0.086
3		15	0.65	153	22.5	123	93	5.33	4.03	0.76	158	0.068
Perfusion pressure average 190 mm. Hg												
4	2.08	2	2.2	158	22.8	14.3	14.1	15.7	15.5	0.99	285	0.111
5		2	2.4	155	21.9	13.2	13.4	15.8	16.1	1.02	289	0.109
6		2	2.6	152	21.9	12.0	11.8	15.6	15.3	0.98	289	0.108
Perfusion pressure average 78 mm. Hg												
7	3.03	15	0.5	137	20.4	161	138	5.37	4.60	0.86	230	0.047
8		15	0.45	136	20.4	164	129.4	4.60	3.87	0.84	228	0.040

In. = inulin; Cr. = creatinine.

#### *Creatinine/inulin clearance ratios during forced diuresis*

These were examined in order to determine whether the creatinine deficit which appeared at low arterial pressures and the consequent low urine flows and high U/P ratios was definitely related to one of these three variables. We have specifically examined these relationships in six experiments. In these a first group of periods at a low arterial pressure yielded a discrepant creatinine/inulin clearance ratio; urea or sulphate diuresis was then induced with consequent removal of the discrepancy; in the third group of periods the perfusion pressure was lowered to a value which restored the urine flow to or below the value obtained in the first group of periods. The third group of experimental periods differed from the first in that it showed much lower U/P ratios and no discrepancy whatever in the creatinine and inulin clearances. A typical experiment

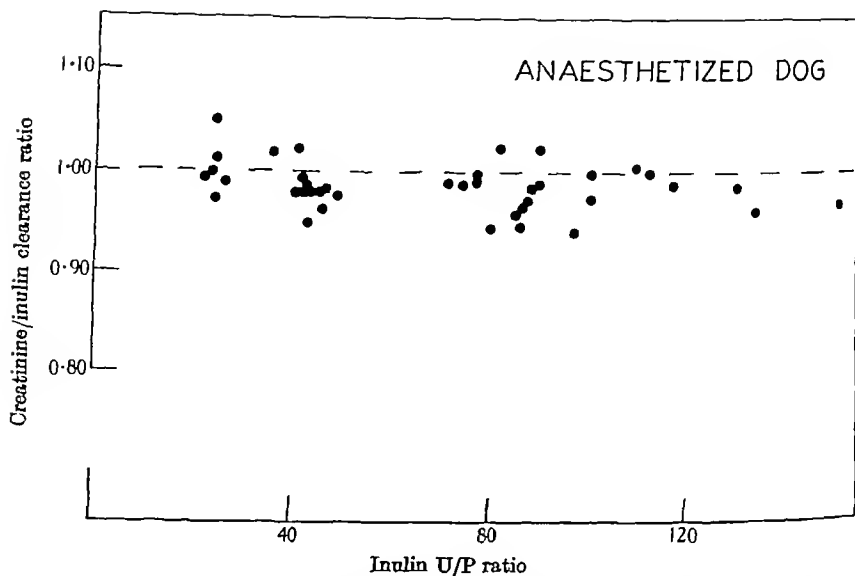


Fig. 1. Chart showing the relation between the creatinine/inulin clearance ratio and the U/P ratio of inulin in the kidney of the anaesthetized dog. Each point represents the ratio of creatinine clearance to the simultaneously determined inulin clearance in a single experimental period. The broken line represents a creatinine/inulin clearance ratio of 1.00.

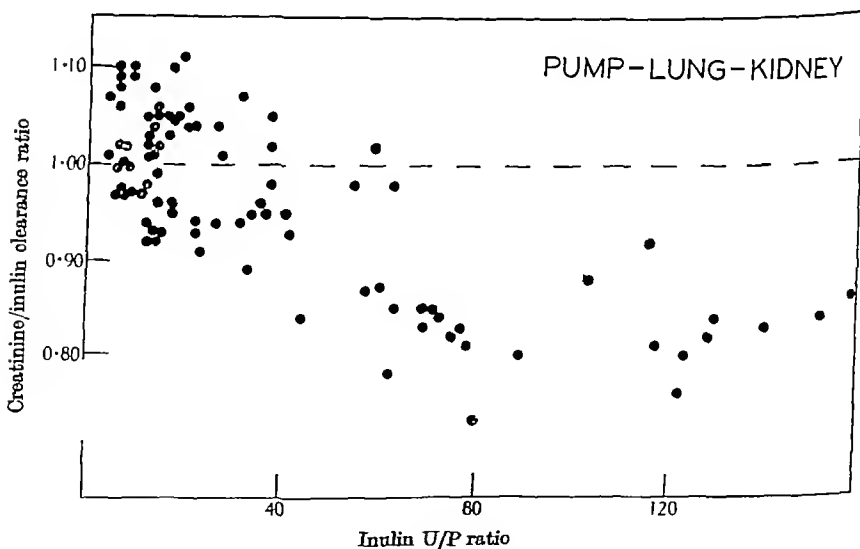


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Period	Time	Dura- tion min.	Vol. of sample c.c.	Plasma level		U/P ratio		Clearance		Clear- ance ratio Cr./In.	Blood flow c.c./ min.	Inulin extrao- tion ratio
				In.	Cr.			In.	Cr.			
				mg. %	mg. %	c.c./ min.	c.c./ min.					
Perfusion pressure average 77 mm. Hg												
1	1.10	15	0.70	161	22.5	129	108	6.01	5.04	0.84	154	0.078
2		15	0.80	156	23.1	124	100	6.61	5.33	0.81	154	0.080
3		15	0.65	153	22.5	123	93	5.33	4.03	0.76	158	0.068
Perfusion pressure average 190 mm. Hg												
4	2.08	2	2.2	158	22.8	14.3	14.1	15.7	15.5	0.99	285	0.111
5		2	2.4	155	21.9	13.2	13.4	15.8	16.1	1.02	289	0.109
6		2	2.6	152	21.9	12.0	11.8	15.6	15.3	0.98	289	0.108
Perfusion pressure average 78 mm. Hg												
7	3.03	15	0.5	137	20.4	161	138	5.37	4.60	0.86	230	0.047
8		15	0.45	136	20.4	154	129.4	4.60	3.87	0.84	228	0.040

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TABLE IV. An experiment showing the difference in the creatinine/inulin clearance ratio at the same urine flow, in the presence and absence of extensive water reabsorption. Exp. 10 C. Weight 7.6 kg. Kidney weight 35.5 g. Blood detoxicated by circulation through lungs 10.30-11.35 a.m. Kidney attached 11.35 a.m. First period started 12.45 p.m. Perfusion fluid was 1 l. of defibrinated blood to which 1.8 g. of inulin and 0.3 g. of creatinine had been added. Throughout the experiment a solution of 4.0% inulin and 0.5% creatinine was added at the rate of 0.2-0.4 c.c./min. The fall in plasma concentrations initially observed is due to irregular additions in the early part of the experiment. The fall of plasma creatinine, however, is the same order of magnitude as that of inulin. 3.6 g. of urea were added at the end of period 3.

Period	Time	Duration min.	Vol. of sample o.o.	Plasma level		U/P ratio		Clearance		Clear- ance ratio Cr./In.	Blood flow c.c./ min.	Inulin extrac- tion ratio
				In. mg. %	Cr. mg. %	In.	Cr.	In. c.c./ min.	Cr. c.c./ min.			
Perfusion pressure average 66 mm. Hg												
1	12.45	16	0.80	170	21.9	115	105	5.75	5.25	0.91	146	0.079
2		16	0.60	150	20.5	140	116	5.25	4.35	0.83	150	0.053
3		16	0.75	127	18.5	129	109	6.03	5.10	0.85	167	0.072
Perfusion pressure average 69 mm. Hg, urea added												
4	1.55	4	2.4	189	25.5	11.80	12.10	7.08	7.26	1.03	164	0.089
5		4	2.6	186	24.7	9.35	10.30	6.08	6.70	1.10	170	0.072
6		4	3.3	182	24.4	8.66	9.39	7.14	7.79	1.09	177	0.081
Perfusion pressure average 52 mm. Hg												
7	2.50	16	0.60	193	24.1	31.0	33.2	1.16	1.24	1.07	146	0.014
8		16	0.50	185	24.1	37.3	39.3	1.16	1.23	1.06	150	0.015

In. = inulin; Cr. = creatinine.

of this kind is described in Table IV. The remaining five gave similar results. It is evident, therefore, that a low creatinine/inulin clearance ratio is essentially associated with a high inulin U/P ratio and not with a low arterial pressure or a low urine flow.

## DISCUSSION

The evidence derived from the observations on normal dogs in favour of the view that the inulin clearance is a measure of glomerular filtration rate applies, so far as the identity of the inulin and creatinine clearances is concerned, with equal force to the anaesthetized dog. This is true also of the isolated kidney at the low U/P ratios, but its significance is somewhat lessened by the fact that there is a discrepancy in the clearances at high U/P ratios in this preparation. In the light of the evidence available on both normal and anaesthetized animals we may safely attribute this discrepancy to the reabsorption of creatinine, or of proportionately more creatinine than inulin, rather than to the secretion of inulin [see also Richards, Bott & Westfall, 1938].

It seems fairly certain that the deficit in the creatinine clearance (i.e. inulin clearance minus creatinine clearance) is not due to the loss of urinary creatinine by a process of simple passive diffusion. The dissipation of a concentration gradient by a process of passive diffusion will be determined by two factors, the properties of the permeable surface and the time available for such diffusion to occur. The tubular permeability may be considered constant under the conditions of the experiments which specifically test whether passive diffusion is the mechanism responsible for the creatinine reabsorption. The time available for diffusion processes to proceed is determined by the rate of flow of tubular urine from its site of entrance to the site of exit from the tubule. When this time is reduced, as in pressure diuresis (Table III) or urea diuresis (Table IV) the creatinine deficit disappears. When the time is increased, however, the deficit does not necessarily reappear, as shown when urea diuresis is counteracted by a reduction in arterial pressure. In Table IV, for example, the time available for diffusion processes to take place was greater in the test periods, where water reabsorption was blocked (i.e. periods 7-8) than in the control periods (i.e. periods 1-3). In the test periods the rate of flow in the proximal portions of the tubules was lower since the rate of glomerular filtration was lower, and in the distal portions, about the same, since the rate of exit (i.e. the urine flow) was about the same. The time for diffusion processes to take place in the test periods was therefore at least as long as in the control periods. It is clear, therefore, that prolonged contact with the tubular wall will not by itself result in the preferential reabsorption of creatinine, that the reabsorption of creatinine occurs only when the tubular reabsorption of water approaches completeness, and that simple passive diffusion is not the mechanism of this loss since the time of contact is not a primary determinant of its extent. We do not suggest that the creatinine reabsorption is active in the sense of glucose reabsorption, but that the creatinine lost may be an incidental constituent of some of the reabsorbed fluid, when the tubular reabsorption of water approaches completeness.

This conclusion suggests that the process of water reabsorption in the isolated kidney is controlled by limiting factors which are different from those in the kidney of the normal or anaesthetized animal. The contrast between the isolated organ and the organ in the anaesthetized animal is shown in Fig. 3, in which the inulin U/P ratio is plotted against the inulin clearance, the latter being varied by variation in perfusion pressure. We cannot accept with confidence the inulin clearance and its

U/P ratio as absolute measures of water filtered, and the fraction reabsorbed in the isolated kidney. However, any error resulting from the reabsorption of inulin would tend to diminish the significance of the contrast portrayed in Fig. 3 rather than be responsible for it. These data demonstrate that in the isolated kidney the fraction of filtered water reabsorbed is largely dependent upon the absolute rate of glomerular filtration; whereas in the anaesthetized dog the rate of filtration appears to be of much less importance in this relation. Two factors are concerned

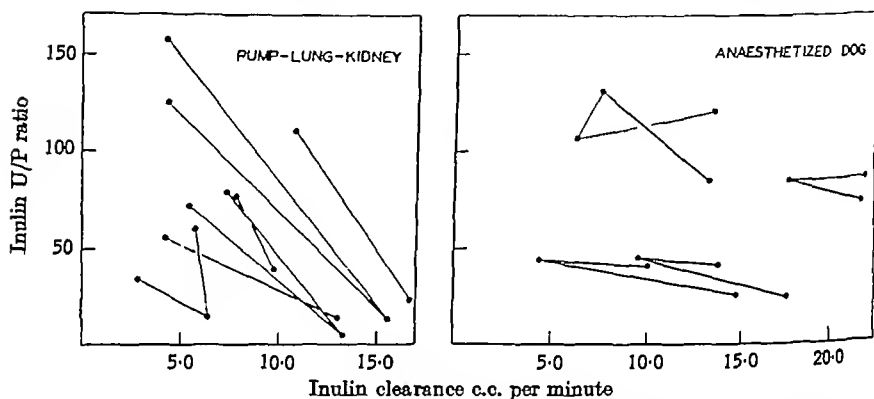


Fig. 3. Chart showing the relationship between rate of water filtration (inulin clearance) and fraction of the total reabsorbed (inulin U/P ratio) as observed in the pump-lung-kidney and the kidney of the anaesthetized dog. Changes in filtration have been produced by changing perfusion pressure. Each point is the mean of two or three consecutive periods observed under a single set of experimental conditions. The lines connect the groups of observations in single experiments in the sequence in which they were obtained.

in determining the fraction of filtered water reabsorbed, first the composition of the plasma, and secondly, the osmotic load in the tubular urine in terms of its total osmotic pressure or that of certain specific osmotically active substances. In the anaesthetized dog the time available at normal filtration rates is sufficient to allow the osmotic load in the tubules to be the preponderating control over the fraction of water reabsorbed, since increasing the time by lowering the rate of glomerular filtration does not greatly increase the final degree of concentration (i.e. the U/P ratio). Similar data have been obtained on the transplanted kidneys of otherwise normal unanaesthetized animals [see Table I, Levy, Robinson & Blalock, 1938]. In the perfused organ with defibrinated blood of normal composition there would seem to be no osmotic factor limiting water reabsorption; this may be due to the hyperactivity of chloride reabsorption

in this preparation [Starling & Verney, 1925]. In such circumstances one might expect a progressive increase in the fraction of filtered water which is reabsorbed, when the time for this process is prolonged by lowering the filtration rate. In the isolated kidney the system is not, therefore, approaching a limiting equilibrium. This process in the isolated kidney may, however, actually arrive at the complete reabsorption of water and contained solutes if the ureter pressure be raised or the arterial pressure lowered far enough [Winton, 1937]. If an osmotic load is placed on an isolated kidney by adding sufficient sulphate or urea to the perfusion fluid and hence to the tubular fluid, a change in the amount of water filtered per unit time has a relatively small effect upon the fraction reabsorbed, as in the kidney in the normal or anaesthetized dog.

To the abnormality of the isolated kidney described by Starling & Verney [1925], i.e. the hypotonicity of the urine, must now be added the two abnormalities described above, the low creatinine/inulin clearance ratios at high U/P ratios and the low values of the absolute inulin clearances. The discrepant creatinine/inulin clearance ratio may be related to the low chloride concentration in the urine for reasons given above, and recalls a similar discrepancy in human patients in diabetic coma described by McCance & Widdowson [1939], especially as in these cases the urine also had an abnormally low chloride content. The low values of the inulin clearances may be due in part to loss of tone of the vasa efferentia, since the blood flow through the isolated kidney is rather high and minimal effective concentrations of adrenaline lower the blood flow but increase the urine flow by constricting the vasa efferentia [Winton, 1931].

#### SUMMARY

1. The extension of the evidence in favour of regarding the glomerular filtration rate as equal to the inulin clearance from normal dogs to anaesthetized dogs and to isolated kidneys has been investigated by comparing the creatinine and inulin clearances.

2. As in normal dogs, the creatinine and inulin clearances are the same in anaesthetized dogs over a range of U/P ratios from 22 to 196 (Fig. 1). In the isolated kidney they are the same only at low U/P ratios, up to about 40, the creatinine clearance falling to about 85% of the inulin clearance in the higher range of inulin U/P ratios of from 65 to 159 (Fig. 2).

3. So far as the identity of the creatinine and inulin clearances is concerned, the evidence for using them as a measure of the glomerular filtration rate is as good in the anaesthetized dog as in the normal animal.

In the pump-lung-kidney their use for this purpose is certainly invalid at high U/P ratios, and possibly so at low U/P ratios.

4. The creatinine clearance is lower than the inulin clearance only when the U/P ratio is high, not when at a correspondingly low arterial pressure and low urine flow the U/P ratio is prevented from rising by increasing the urea content of the blood. This, it is argued, precludes the creatinine deficit being solely due to reabsorption by passive diffusion.

5. The influence of arterial pressure on the clearance is not very different in the two preparations, 10 mm. Hg producing about an 8% change in the clearance in the normal range of arterial pressure (about 120 mm. Hg).

6. A given change in clearance produces three or four times the change in inulin U/P ratio in the isolated kidney than it does in the denervated kidney *in situ* (Fig. 3) unless urea or sulphate is acting upon the former. This is attributed to the abnormally low osmotic load of the tubular contents due to overactivity of chloride reabsorption in the isolated kidney.

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## CAPILLARY PERMEABILITY AND OEDEMA IN THE PERFUSED FROG

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### INTRODUCTION

If the hindlegs of a frog are perfused with 3% gum acacia in Ringer solution, the capillaries dilate, and the capillary walls become permeable to protein, so that a rapid oedema develops. The melanophores contract also, and no doubt other changes occur in the tissues, since perfusion tends to remove the whole of the intricate system of hormones, enzymes, etc., of the plasma which maintain normal equilibrium. In this paper attention is focused on the nature of the change in capillary permeability. Krogh [1929] has taken the attitude that capillary dilation is a phenomenon essentially linked with increased permeability; but on this point, as on most of the other points concerned, the evidence in the literature is contradictory.

In 1921 Krogh & Harrop found that the addition of defibrinated ox blood to 3% acacia prevents capillary dilation. Krogh [1924] suspected that pituitrin was responsible for maintaining normal capillary diameter and permeability. Dale [1926] took the contrary view. Drinker [1927] found that pituitrin had no action in preventing dilation and oedema, but that with 20% horse serum added to 3% acacia, both dilation and oedema were prevented. Krogh [1929], using a 33% suspension of ox red cells in 3% acacia, found that the capillaries are dilated, but that there is no oedema. Saslow [1938] confirmed Drinker's observation that serum will prevent dilation, but could not confirm Drinker's observation that oedema is also prevented. Saslow found that 19-24% ox red cells in acacia will stop oedema but not dilation, and that with red cells in serum neither oedema nor capillary dilation are found.

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In view of these contradictory observations it is necessary to restudy the field completely.

### THEORETICAL CONSIDERATIONS

Landis [1934] has shown that in the frog there is a considerable fall in pressure between the arteriolar and venular ends of the capillary network, and the c.o.p. (colloid osmotic pressure) of frog serum lies between the pressures at the two ends of the network, as is demanded by Starling's [1896] hypothesis of the relations between perfusion pressure, colloid osmotic pressure and lymph formation. At the arteriolar end of the capillaries under these conditions, a fluid containing all the non-colloidal constituents of the serum is filtered out through the capillary wall, under the driving force of the difference between the pressure in the capillary, and the c.o.p. of the serum. Over the venular end of the capillaries, where the capillary pressure is less than the serum c.o.p., fluid returns to the capillaries. According to Churchill, Nakazawa & Drinker [1927], the capillaries of the frog are normally slightly permeable to protein, so that complete recovery of the lymph does not occur at the venular end of the capillaries, and the residuary lymph is returned to the general circulation by the lymph hearts. We shall now obtain a general relationship between the c.o.p. of the serum, the perfusion pressure, etc., and the permeability of a capillary system slightly permeable to protein.

Assume that the capillary is a tube  $XZ$  of uniform diameter and of length  $l$  (Fig. 1). At  $Y$  the c.o.p. of the serum is equal to the capillary pressure. The distance  $XY$  is  $l'$ : Assume also that the protein-permeable pores are evenly distributed through the whole length of the capillary. Then for a homogeneous solution the pressure gradient through the capillary will be linear in the state of steady flow. Let

$R$  = rate of accumulation of fluid in the tissue;

$R_1$  = rate of filtration of fluid through pores permeable to protein;

$R_2$  = rate of filtration through remaining pores over the length  $XY$ ;

$R_3$  = rate of back-filtration through remaining pores over the length  $YZ$ ;

$R_4$  = rate of back-filtration due to return of protein from the lymph to the capillaries;

$P_a$  = pressure at the arteriolar end of the capillaries;

$P_v$  = pressure at the venular end of the capillaries;

$p$  = c.o.p. of the serum;

$p'$  = c.o.p. of the lymph at  $Z$ .

Assume also that the flow through a pore is proportional to the pressure difference between the two ends of the pore. Then we have

$$R_1 = \left( \frac{P_a + P_v}{2} \right) lA, \quad (1)$$

where  $A$  is a constant.

$$R_2 = \left( \frac{P_a - p}{2} \right) l'B,$$

but

$$\frac{l'}{P_a - p} = \frac{l}{P_a - P_v},$$

$\therefore$

$$R_2 = \frac{(P_a - p)^2}{2(P_a - P_v)} lB, \quad (2)$$

where  $B$  is a constant. It is assumed here that the rate of filtration of protein is low, so that the value of the c.o.p. of the lymph may be

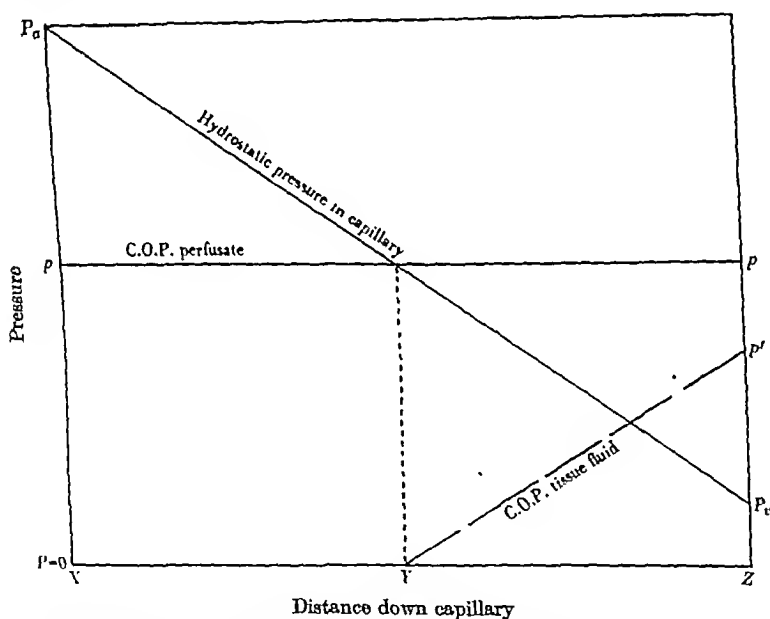


Fig. 1. Diagram showing relationships between the hydrostatic pressure,  $P$ , in a capillary and the colloid osmotic pressure,  $p$ , and  $p'$ , of the blood and intercellular fluid.

neglected over the length  $XY$ . Over the length  $YZ$  the c.o.p. of the lymph is assumed to rise linearly from zero at  $Y$  to a value  $p'$  at  $Z$ . This will be approximately true if  $R_1$  is small compared with  $R_2$ , and  $p$  is not very large. Then

$$\begin{aligned} R_3 &= - \left[ \frac{p - P_v}{2} - \frac{p'}{2} \right] (l - l') B \\ &= -(p - P_v - p') \left[ 1 - \frac{P_a - p}{P_a - P_v} \right] \frac{lB}{2}, \end{aligned} \quad (3)$$



assuming that the rate of filtration is the same in passing from lymph to serum as from serum to lymph (i.e.  $B$  is the same constant in both cases). In obtaining  $R_1$ ,  $R_2$ ,  $R_3$ , the variation of  $p$  with distance down the capillary has been neglected.

$R_4$  will be mainly determined by the diffusion coefficient of the colloids, and will be very small. Judging from the observations of Landis [1927, 1928] the rate is so small as to be negligible, so that

$$R_4 = 0. \quad (4)$$

Then the rate of accumulation of fluid is

$$R = R_1 + R_2 + R_3 + R_4 \\ = \left( \frac{P_a + P_v}{2} \right) lA + \frac{(P_a - p)^2}{2(P_a - P_v)} lB - (p - P_v - p') \left[ 1 - \frac{P_a - p}{P_a - P_v} \right] \frac{lB}{2}. \quad (5)$$

(5) could be simplified if there were a general relationship between  $p$  and  $p'$ . Under the conditions of the experiments reported here this appears to be the case. For haemoglobin perfusates containing from 2 to 7% Hb, the concentration of haemoglobin in the oedematous fluid found between the skin and muscles after a long perfusion was found, by colorimetric methods, to contain about half the amount of haemoglobin in the perfusate. This suggests that there is a linear relationship between  $p$  and  $p'$ . But  $p'$  cannot be greater than  $(p - P_v)$ , and when  $p = P_v$ ,  $p' = 0$ . Hence, if the relationship is linear we have  $p' = k(p - P_v)$ , where  $k$  is a constant. Then (5) becomes

$$R = \left( \frac{P_a + P_v}{2} \right) lA + \frac{lB}{2(P_a - P_v)} \{kp^2 - p(2P_a - 2P_v + 2kP_v)\} + \frac{lB(P_a^2 - P_v^2 + kP_v^2)}{2(P_a - P_v)} \\ = xp^2 - yp + z, \quad (6)$$

where

$$x = \frac{lBk}{2(P_a - P_v)}, \\ y = \frac{lB}{2(P_a - P_v)} [2P_a - 2P_v + 2kP_v], \\ z = \left( \frac{P_a + P_v}{2} \right) lA + \frac{lB(P_a^2 - P_v^2 + kP_v^2)}{2(P_a - P_v)},$$

$x$ ,  $y$ , and  $z$  are constants if  $P_a$ ,  $P_v$ ,  $A$ ,  $B$ , and  $k$  are constant. Equations (5) and (6) will not be true for large values of  $p$ .

In deducing (5) and (6), two assumptions, that  $p' \approx 0$ , and that  $p$  is independent of  $l$ , have been made; these cannot simultaneously be true unless the total interchange of fluid through the capillary walls is small compared with the rate of flow from one end of the capillary to the other. If  $p$  is small the rate of oedema development is determined mainly by the term in equation (6) which does not contain  $p$ , and hence compliance with these assumptions is relatively unimportant. But when  $p$  is large the

terms in  $p$  and  $p^2$  are important, and these assumptions also attain increasing importance. In the limiting case where  $p$  is very large and the rate of development of oedema is very large, equation (6) must break down. Rough calculations suggest that, in terms of the units of c.o.p. used in this paper (given later), this source of error becomes serious for values of  $p$  of 10 or more.

If the capillaries are impermeable to protein,  $p' = 0$ , and  $A = 0$ , in which case (5) reduces to

$$R = \left( \frac{P_a + P_r}{2} \right) lB - lBp. \quad (7)$$

From a comparison of (6) and (7) it will be seen that permeability to protein introduces extra terms in  $p$ , and a new term in  $p^2$ . Frog capillaries are not sufficiently impermeable to protein and resistant to high values of  $P_a$  for equation (7) to be tested, but equation (6) will be used in the present paper. Agreement with (6) shows that the rate of development of oedema is compatible with the simple physical processes described here. But since (6) contains three arbitrary constants, such agreement cannot be taken as *proof* that the present analysis corresponds to the details of the processes occurring in the tissue.

When perfusing with red cells it is possible that a fresh factor enters into the resistance observed, particularly with contracted capillaries. This is friction between the red cells and the capillary walls, such as would be met by passing marbles through a tube of their own diameter. This frictional effect cannot be accounted for by measuring the bulk viscosity of the perfusate. The pressure-gradient curve would, strictly, present a series of steps, but if the average gradient is measured over a considerable length of capillary the pressure gradient will again be linear, and equations (6) and (7) will hold for this system also.

#### METHODS, ETC.

Landis [1927] has used a quantitative method for measuring the permeability of individual capillaries, which, however, was unsuitable for the experiments contemplated here. Drinker [1927] devised a qualitative method of detecting oedema which was also unsuitable. In the present work it was found that accurate and rapid measurements could be made by weighing a preparation of the hindlegs of the frog at intervals during perfusion. Hungarian frogs were used. The abdominal contents were removed, with the exception of the kidneys, a cannula inserted in the dorsal aorta, and the frog was traused just above the anterior bifurcation of the aorta, the head half being removed. The nerves running to

the legs were usually cut, though not always. The perfusion fluid was allowed to issue from the kidneys, the abdominal vein, or both. The arterial pressure was maintained at 14 cm. and the venous pressure at 2-3 cm. This means that if the perfusion fluids are inert, not causing changes in the diameter of the vessels, the flow should vary according to the viscosity of the perfusion fluid, and the pressure gradients in the circulatory system should be constant. Consequently  $P_a$  and  $P_v$  should also be constant. Such conditions were found with, for example, gum acacia, but not with, for example, serum.

The Ringer solution used contained 6.75 g. NaCl, 0.15 g. KCl, 0.2 g. CaCl, 0.02 g.  $\text{Na}_2\text{HPO}_4$  per litre. Ox serum was centrifuged for 10 min. at 2000 r.p.m. to remove red cells. It was stored either at 2 or at  $-10^\circ\text{C}$ . Samples from about ten different animals were used. Serum prepared in this way contains a considerable quantity of platelet material. Gum acacia was prepared by dissolving in Ringer solution, and dialysed against large volumes of Ringer, then filtered, after which the pH was adjusted to 7.2. Ovalbumin was crystallized five times by the method of Larosa [1927], and dialysed against Ringer solution. Ox haemoglobin was prepared by washing red cells twice with Ringer solution, followed by haemolysis, either osmotic or by freezing and thawing. Such haemoglobin contains a considerable quantity of more or less disintegrated platelets, and the stroma and contents of the red cells, but only a trace of serum proteins. Part of the stroma, etc., could be removed by centrifuging for long periods. Sheep haemoglobin was prepared either by the simple osmotic process used for ox haemoglobin, or crystallized four times by the method of Adair & Adair [1934]. The latter method gave a comparatively clean preparation. Platelets were obtained from horse blood by a method similar to that of Code [1937]. Horse blood was run into a waxed vessel containing sufficient 10% potassium oxalate to make the solution 0.15% in oxalate. The red cells were allowed to settle, the plasma decanted off and centrifuged for 5 min. at 2000 r.p.m. to remove white cells, then for 40 min. at 4000 r.p.m. to bring down the platelets, which were resuspended in a suitable fluid. Heparin was used as an alternative anticoagulant.

Oxygen was continuously bubbled through perfusion fluids unless otherwise stated.

## RESULTS

*Ox serum.* Ox serum has a tonicity 1.37 that of frog serum, and a c.o.p. 2.5 times that of frog serum. Fig. 2 shows typical results for frogs perfused with various percentages of ox serum in Ringer, after adjustment of tonicity by addition of water. For the perfusates the percentages

refer to the percentage of normal ox serum colloids in the diluted serum. In the figure percentage by weight of the initial weight of the preparation is plotted against time. When Ringer solution only is used a very rapid oedema sets in, and only stops when the tissue spaces are completely filled, and the tissue becomes turgid; this occurs after a 40-60% increase in weight. With the addition of serum the rate of increase in weight diminishes rapidly, quite out of proportion to the c.o.p. of the serum, as is shown by the fact that oedema is reduced a hundredfold by 40% serum,

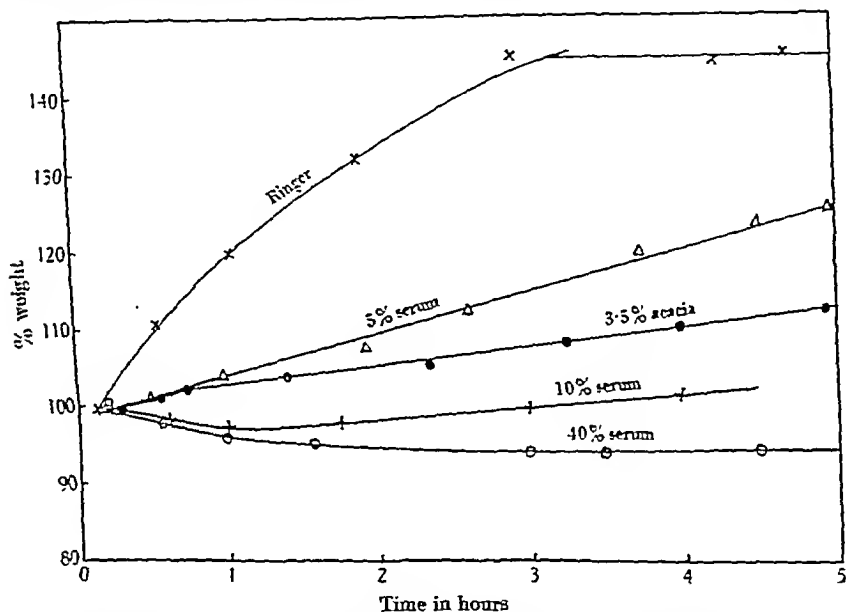


Fig. 2. Diagram showing the development of oedema with time during perfusion with Ringer solution, various dilutions of ox serum, and 3½ % acacia in Ringer solution.

but only tenfold by 3.5% gum acacia; both have about the same c.o.p. The rate of development of oedema as increase in weight per minute, in per cent of the initial weight, is given in Table I: the results recorded in this table, and in Fig. 1, were obtained with autumn frogs. Spring frogs usually showed a greater permeability to colloids. The results shown in Table I are not compatible with equation (6).

It will be seen (Fig. 2) that during the first 40 min. of perfusion there are several changes of slope in percentage-weight curves. These are probably due to a number of causes, such as dilation and contraction of the vascular system, the taking up or loss of water by the lymph initially present in the tissues, and possibly the washing of potassium out of the

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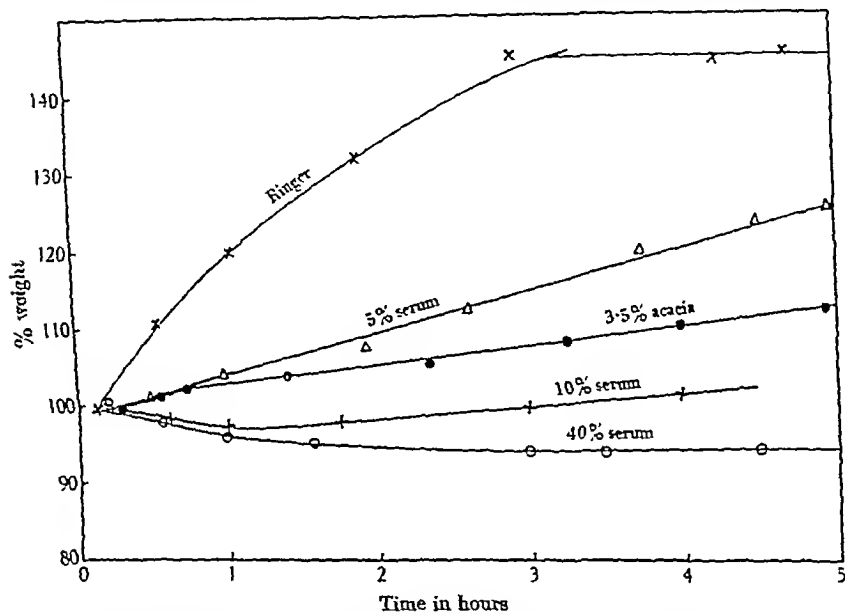


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TABLE I. Effect of adding ox serum to Ringer solution on rate of oedema

% serum colloids in perfusate	Rate of oedema formation, %/min.	C.O.P. of perfusate as % frog serum C.O.P.
0	0.48	0
5	0.1	12.5
10	0.03	25
30	0.01	75
40	0.003	100
3.5 % acacia	0.04	100

tissues, since Horton [1930] found that operative procedures may produce a species of shock, in which the muscles may lose large amounts of potassium. No doubt the cells of the tissues also participate to some extent in the water equilibrium.

TABLE II. Rate of development of oedema with mixtures of ox serum and gum acacia of the same C.O.P. as frog serum

Perfusate	Rate
3.5 % acacia	0.26
2.6 % acacia + 10 % serum	0.18
1.75 % acacia + 20 % serum	0.12
1.3 % acacia + 25 % serum	0.04
0.88 % acacia + 30 % serum	0.01
40 % serum	0.005

Table II shows the rates of oedema development with various mixtures of serum and acacia, all approximately of the same C.O.P. as frog serum; the results were obtained with spring frogs. It will again be seen that serum is far more effective than gum acacia in preventing oedema. This confirms the results of Drinker [1927], but is contrary to the observations of Saslow [1938]. It may be concluded that in the presence of sufficient serum the capillaries are only slightly permeable to gum acacia and to serum albumin, but are much more permeable to these colloids in the absence of sufficient serum.

Oedema developed at about the same rate when serum was brought into equilibrium with air and with oxygen. A large supply of available oxygen is not necessary for maintaining normal permeability with resting tissue. This is contrary to the observation of Saslow [1938].

*Gum acacia.* There is a considerable variation in the rate of oedema development with different frogs; with autumn frogs a minimum rate of 0.04 % was found with 3.5 % acacia, but with spring frogs values as high as 0.29 % were recorded.

In Table III are given a typical set of values of the rate of oedema formation with various concentrations of acacia. For simplicity in comparing the constants of equation (6) for different substances the C.O.P.

TABLE III. Results of perfusion with various amounts of acacia in Ringer solution

Acacia %	<i>p</i> osmotic equivalent	C.O.P. perfusate as % C.O.P. serum	<i>R</i> observed	<i>R</i> calculated
1.75	0.625	50	0.31	0.32
3.5	1.25	100	0.22	0.23
5.25	1.875	150	0.17	0.15
7.0	2.5	200	0.10	0.08
8.75	3.125	250	0.015	0.015

has been expressed as an "osmotic equivalent", in units of the C.O.P. due to 1% of a substance with molecular weight 34,500, i.e.

$$p = C \times \frac{34,500}{M},$$

where *C* is the concentration of the colloid in per cent by weight, and *M* is its molecular weight. The calculated values of *R* given in the last column were obtained from the equation  $R = 0.395 - 0.14p + 0.005p^2$ . The observed and calculated values are in reasonable agreement, so that it may be concluded that with gum acacia perfusates the preparation appears to behave as a simple physical system.

*Ovalbumin.* Hen's ovalbumin, purified by crystallization and dialysis, behaves similarly to gum acacia. The results obtained by perfusion of the same frog with different amounts of ovalbumin in Ringer solution are shown in Table IV. The calculated values of *R* were obtained from the equation  $R = 0.485 - 0.14p + 0.011p^2$ , and are in reasonable agreement with the observed values.

TABLE IV. Results of perfusion with Ringer solution containing various amounts of crystallized ovalbumin

% ovalbumin	C.O.P. as % serum C.O.P.	<i>R</i> observed	<i>R</i> calculated
0	0	0.485	0.485
0.73	58	0.38	0.39
2.2	176	0.2	0.23
7.3	580	0.06	0.03

*Ox and sheep red cells.* When changing from perfusion with, for example, 3.5% acacia, with which the capillaries are permeable to colloid, to a similar perfusate containing red cells, fluid sometimes filters out so rapidly from the red cell suspension that the capillaries become blocked by a solid mass of red cells. This condition of stasis is accompanied by a very large drop in the rate of flow. Such experiments have been discarded, and those referred to here are cases in which the rate of flow changes in a reversible manner on passing from one perfusate to another.



Saslow's experiment, perfusion with 25% ox red cells in acacia, was repeated and confirmed. In a typical experiment perfusion was started with 25% red cells in 3.5% acacia; the rate of oedema  $R$  was 0.039%/min. On changing to the same acacia solution without red cells,  $R$  rose to 0.21%/min.; on returning to 25% red cells,  $R$  fell to 0.033%/min. Adding 25% red cells to acacia is quite effective, reducing the rate of development of oedema to between one-sixth and one-tenth of its rate with acacia alone; if this experiment is compared with that in Table II, in which serum was added to acacia, it will be seen that 25% red cells have about the same effect as 25% serum. This coincidence proved to be fortuitous, for, as the protocol of the typical experiment of Table V shows, 2.5% red cells is just as effective as 25% in reducing the rate, and  $1\frac{1}{4}\%$  is not greatly inferior in its action.

TABLE V. The effect of adding oxygenated, and partly oxygenated, ox red cells to gum acacia, on the rate of oedema. Red cells fully oxygenated, unless otherwise stated

Perfusate	Rate %/min.
3.5 % acacia	0.14
3.5 % acacia + 2.5 % red cells	0.015
3.5 % acacia + 2.5 % red cells, half-reduced	0.05
3.5 % acacia + 2.5 % red cells	0.017
3.5 % acacia + $1\frac{1}{4}\%$ red cells	0.04

These results certainly agreed with Saslow's view, that red cells maintain normal permeability by providing a plentiful supply of oxygen, although in the experiments with ox serum it was found that comparatively little oxygen was required. Even with  $1\frac{1}{4}\%$  red cells the oxygen content of the venous fluid never reached a level as low as half that of the arterial fluid, so it was decided to try the effect of perfusion with half-reduced red cells. Red cells were reduced by bubbling nitrogen through a 2.5% suspension in gum acacia, and then mixed with an equal volume of 2.5% oxygenated cells. With this half-reduced mixture the rate of oedema did increase, to about the same level as was found with  $1\frac{1}{4}\%$  oxygenated cells (Table V). Thus the effectiveness of red cell suspensions in reducing  $R$  is at least roughly proportional to their oxygen-carrying capacity, for 2.5% red cells half-reduced carry the same amount of oxygen as  $1\frac{1}{4}\%$  fully oxygenated, though, of course, in the former case it is not quite so readily available.

But it was noticed at the same time that bubbling large quantities of nitrogen through a red cell suspension does more than change the oxygen content of the fluid. For the first half-hour the bubbles form a rather stable foam, but after an hour or two the foam is very unstable

and has an "oily" appearance. This is not due to lysis of the red cells. Evidently some substance or body present in the red cell suspension is altered or destroyed by bubbling with nitrogen, and this factor must be eliminated before the action of red cells can be ascribed to their oxygen-carrying powers.

On turning to sheep red cells further evidence was found that the action of red cell suspensions is not due to their power of carrying oxygen. Table VI shows data for red cells from two different sheep.

TABLE VI. The effect of addition of sheep red cells to 3.5 % acacia, using washed cells from two different sheep

Perfusate	Sheep 1	Sheep 2
3.5 % acacia	0.14	0.21
3.5 % acacia + 2.5 % sheep cells	0.17	0.19
3.5 % acacia + 5 % sheep cells	0.14	0.12
3.5 % acacia + 10 % sheep cells	—	0.014
3.5 % acacia + 2.5 % ox cells	—	0.025

There were minor differences between the activities of the two lots; similar differences were found with ox bloods. The general conclusion to be drawn from Table VI is that sheep red cells up to 5 % have little action in reducing *R*, 10 % of cells has quite a marked action, and that, volume for volume, ox cells are three to four times as active as sheep cells. The results of Hill & Wolvekamp [1936] show that ox cells and sheep cells have rather similar  $O_2$  pressure—%  $HbO_2$  curves, and ox and sheep cells have about the same oxygen-carrying capacity. It follows that the difference between ox and sheep cells cannot be due to differences in their oxygen-carrying capacity.

*Haemolysed red cells.* The results obtained by perfusion with Ringer solution containing various amounts of crude haemoglobin from osmotically haemolysed red cells are given in Table VII. The rate of development

TABLE VII. Rate of oedema with various amounts of crude ox haemoglobin in Ringer solution

% haemoglobin	Osmotic equivalent, <i>p</i>	C.O.P. as % C.O.P. serum	<i>R</i> observed	<i>R</i> calculated
0	0	0	0.5	(0.127)
1.0	0.4	40	0.1	0.102
2.5	1.0	100	0.06	0.071
3.5	1.4	140	0.042	0.054
6	2.4	240	0.03	0.030
7	2.8	280	0.025	0.026

of oedema is in all cases greater than is found when the same amount of haemoglobin is present as red cells, even when gum acacia is added to the lower concentrations of haemoglobin to bring the C.O.P. up to that of

frog serum. Fig. 3 shows a typical case. The rate of oedema with 3.5% acacia is 0.25%/min. On changing to (0.75% haemoglobin+2.5% acacia), which has the same osmotic pressure as 3.5% acacia,  $R$  falls to 0.125, but on changing to (3.5% acacia+2.5% red cells), which contains the equivalent of 0.75% of haemoglobin, the rate falls to 0.0125%/min.; i.e. a given amount of red cells is far more effective than the same amount of red cells after haemolysis. On returning to (2.5% acacia+0.75% haemoglobin),  $R$  returned to 0.08%/min. If the haemoglobin is obtained by repeated freezing and thawing of corpuscles, there is a similar loss of ability to reduce the rate of oedema.

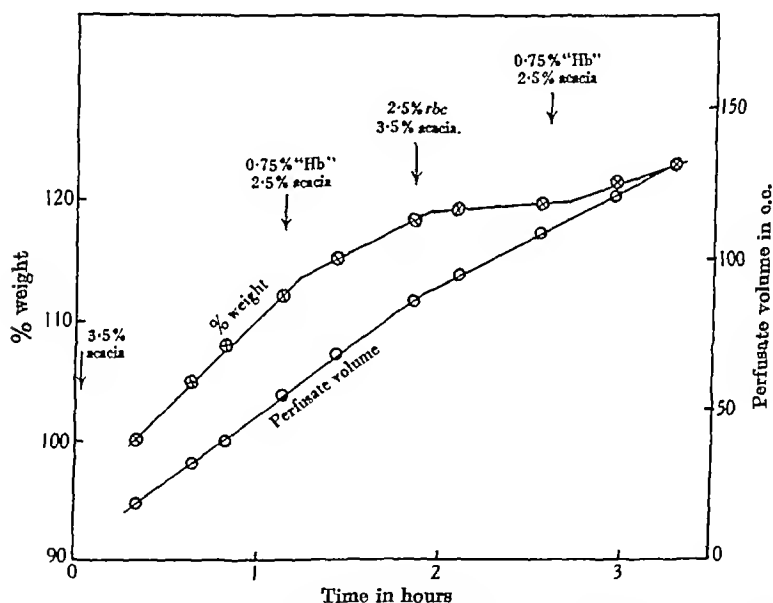


Fig. 3. Comparative action of haemoglobin when added to gum acacia (a) as red cells (*rbc*), (b) as haemoglobin (Hb); ⊗ % weight, ○ total volume of perfusate in c.c.

At first sight it would appear that the partial loss, due to haemolysis, of ability to prevent oedema is sufficient proof that the oxygen-carrying capacity of the red cells is not involved. But it has been shown by Anrep, Barsoum, Talaat & Wieninger [1939] that red cells may contain a histamine-like substance. On haemolysis this is released from the red cells, and may have a dilating action on the capillaries, thus antagonizing any possible contraction produced by the oxygen-carrying powers of the haemoglobin. It is therefore evident that purified haemoglobin must be used.

Returning to Table VII, it is interesting to observe that, with this crude "haemoglobin", the rate of oedema development decreases far more rapidly than can be accounted for by the simple osmotic action of the "haemoglobin", although, of course, not as rapidly as for the same volume of red cells in suspension in a medium of the same C.O.P. Thus equation (6) will not fit the results. But it will fit the results for all concentrations of "haemoglobin" greater than 1%. It is as though the first 1% of "haemoglobin", in addition to its osmotic activity, has a further action by which it reduces the rate of oedema with  $p=0$  from 0.5 to 0.127%/min. After this the results fit the equation

$$R = 0.127 - 0.067p + 0.011p^2,$$

as is shown by the last column of Table VII.

The reduction in rate of oedema due to crude haemoglobin is far less than that due to an osmotically equivalent amount of ox serum.

Crude sheep haemoglobin in a few preliminary experiments appeared to be similar to ox haemoglobin, except that it is still less effective in reducing the rate of oedema.

*Crystallized sheep haemoglobin.* Table VIII shows one series of results obtained with crystalline sheep haemoglobin. About 15% of the haemoglobin was converted into methaemoglobin by the purification process,

TABLE VIII. Rate of oedema with various amounts of crystallized sheep haemoglobin in Ringer solution

% Hb	Osmotic equivalent, $p$	C.O.P. as % C.O.P. serum	$R$ observed	$R$ calculated
0	0	0	0.49	0.49
1.33	0.665	53	0.41	0.4
2.66	1.33	106	0.3	0.31
5.33	2.66	213	0.23	0.19
8.0	4.0	320	0.15	0.10
16.0	8.0	640	0.06	0.07

so that the oxygen-carrying power of the solutions is about 15% less than that of corresponding crude haemoglobin solutions. Even when allowance is made for this, it is evident that at a given colloid osmotic pressure purified haemoglobin is far inferior to crude haemoglobin in ability to reduce oedema. In fact, the pure haemoglobin is identical with ovalbumin if solutions of the same C.O.P. are compared, and the results obey the same equation:  $R = 0.49 - 0.14p + 0.011p^2$ . It thus seems reasonable to suppose that haemoglobin, when pure, can reduce the rate of oedema formation by virtue of its colloid osmotic pressure only. The oxygen-carrying powers of red cells and haemoglobin must play, at most, a

very minor role in maintaining normal permeability. There is, of course, no doubt, from the results of Landis [1928], that severe oxygen lack does cause increased permeability, but it is quite certain that the capillaries of resting tissue need only a very moderate amount of oxygen, such as can be conveyed in ordinary serum.

*Platelets, etc.* From the foregoing results the following conclusions emerge:

(1) Ox serum, freed from red cells by moderate centrifuging, has a large action in reducing capillary permeability. Horse serum is also active [Drinker, 1927].

(2) Red cells, after freeing from serum by centrifuging and washing, have about ten times the activity of serum. Thus activity is not due to the oxygen supply provided by the red cells.

(3) Haemolytic procedures caused a partial loss of activity, and purification of the haemoglobin causes a complete loss of activity other than that due to the C.O.P. of the haemoglobin.

It seems fairly clear from this that the active factor must be one of the formed bodies of the blood, which may be concentrated along with the red cells by centrifuging. Saslow's observation, that serum prevents dilation but not oedema, at first appears contradictory, but he states that his serum was sterilized by filtration through a Berkefield candle. This procedure will remove the platelets, white cells, etc., as well as the bacteria. So Saslow's observations actually direct still further attention to the formed bodies of the serum. Suspicion naturally centres on the platelets.

Accordingly, suspensions of horse blood platelets, and of white cells and platelets were prepared in  $3\frac{1}{2}\%$  gum acacia. It was found that preparations containing less than 0.025 c.c. of platelets in 100 c.c. of  $3\frac{1}{2}\%$  acacia reduced the rate of oedema to an extent comparable with 25% serum, or 2.5% ox red cells; i.e. the platelet preparations were at least a hundred times more active than red cells and a thousand times more active than serum. This activity was lost if the platelets were allowed to clot completely. Preparations from oxalated plasma frequently reduced the rate of flow by 90%, but heparin preparations had little effect on this. Fig. 4 shows a typical experiment. The perfusion was started on  $3\frac{1}{2}\%$  acacia; at *A* the perfusate was changed to acacia to which "clotted platelets" had been added; there was practically no change in either the rate of flow or the rate of oedema. This "clotted platelet" suspension was made by adding acacia to platelets centrifuged down from plasma, followed by gentle stirring. Several large clots formed, and very little,

if any, material went into suspension. At *B* the perfusate was changed back to acacia, and at *C* to a suspension of platelets obtained from heparinized plasma. After about 10 min. the preparation actually began to lose water; this loss of water would not have continued indefinitely, but resembles the changes found when serum is used for a perfusion, and it would eventually have given place to a slow increase in weight. The loss of weight is probably due to the fact that the "platelets" greatly

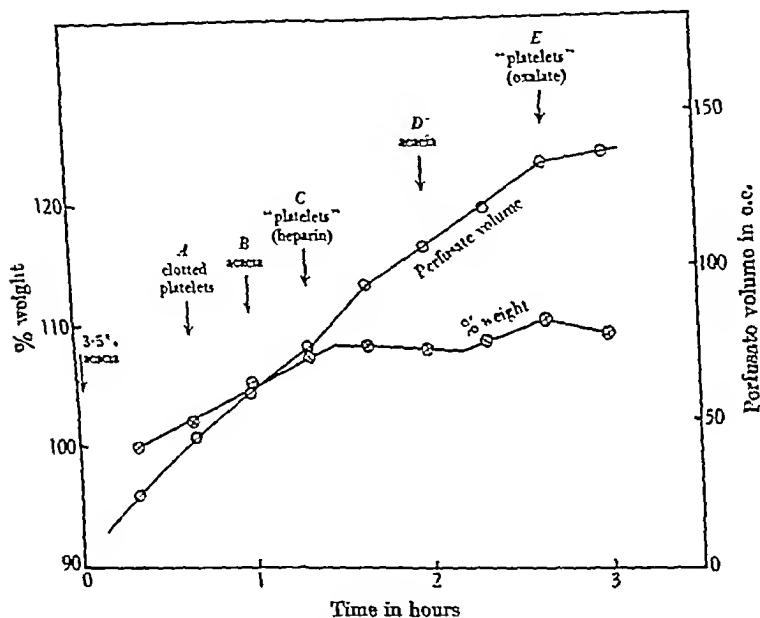


Fig. 4. The effect of adding (a) clotted platelets, (b) unclotted platelets, to 3½ % acacia.  
⊗ % weight, ○ total volume of perfusate in c.c.

reduce the permeability of the capillary to colloids, and a fresh equilibrium has to be set up with the lymph, which, since the capillary has become a more efficient apparatus, involves the absorption of water by the plasma. Such platelet suspensions were obtained by adding acacia to centrifuged platelets, followed by violent mechanical stirring; under these conditions part, but not all, of the platelets clot, and may be removed by gentle centrifuging, or by allowing to stand for half an hour; such suspensions are slightly opalescent. At *D* the perfusate was changed back to acacia; after about 15 min. the preparation began to gain water again. At *E* a suspension of oxalated platelets was used; this again caused a loss in weight, but the rate of flow was cut down by 80%, so that this

last result is difficult to interpret. It seems probable that oxalated platelets tend to clog the capillaries.

It will be noticed that, on changing from or to a solution containing platelets, the full activity of the platelets is not evident until 10–15 min. after the change is made. This contrasts sharply with changes from a fluid of one C.O.P. to one of another C.O.P., when the change in rate of oedema is complete in 3–4 min. at most.

*Effect of addition of clupein to serum and to acacia*

Addition of 0.1 % by weight of clupein to 3½ % gum acacia reduces the rate of flow by about 50 %, and reduces the rate of development of

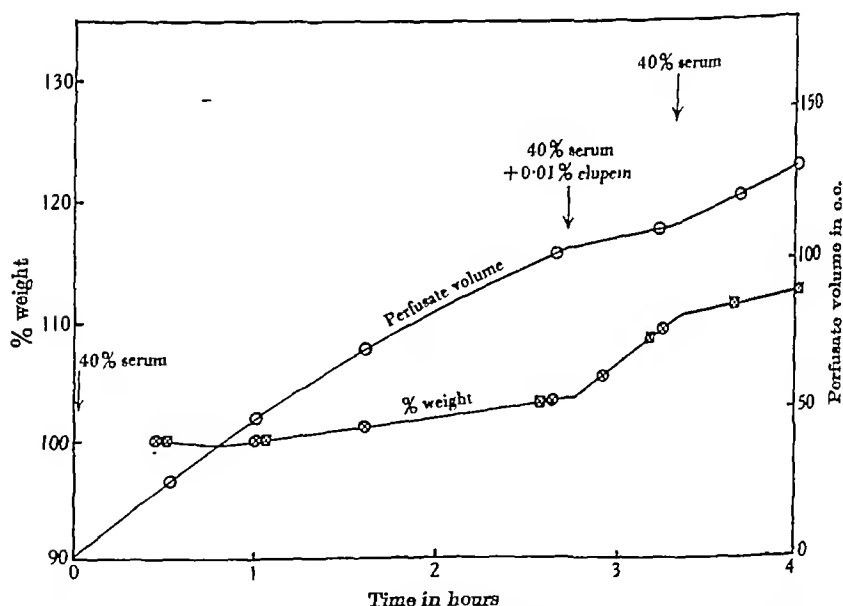


Fig. 5. Effect on rate of development of oedema of addition of 0.01 % clupein to 40 % serum. ⊗ % weight of first preparation, ⊗ % of weight of second preparation. ○ total volume of perfusate, first preparation.

oedema by about the same amount. It seems probable that the reduction in the rate of flow is due to contraction of part at least of the vascular system, and that the reduction in rate of oedema may be partly due to the increase in colloid osmotic pressure produced by the clupein, and partly to capillary contraction. This, however, was not further investigated.

When added to 40% serum 0.1% clupein produces a precipitate, but 0.01% clupein forms a clear solution, provided the clupein is added to the serum in solution. Solid clupein does not readily dissolve in serum. Two experiments are illustrated in Fig. 5; the two experiments were made with different frogs, but otherwise were carried out in precisely the same way. The perfusion was carried out with 40% serum for about  $2\frac{1}{2}$  hr., during the later  $1\frac{1}{2}$  of which the rate of development of oedema is small and constant. Change was then made to serum containing 0.01% clupein: as with acacia, the rate of perfusion was reduced. But the rate of oedema was markedly increased, from 0.036 to 0.19%/min. On returning to 40% serum alone, the rate of perfusion rose again, and the rate of oedema fell to 0.05%. Thus the effect of clupein is partially, but perhaps not completely, reversible.

It is possible that this action of clupein in increasing the rate of oedema in the presence of proteins is due to enlargement of capillary pore size by displacement of adsorbed serum proteins: a possible mechanism for this will be discussed later. If this explanation is not correct, then clupein should prove useful in analysing the exact mechanism by which serum, including platelet material, restricts capillary permeability.

#### DISCUSSION

It is evident from the results given here, that with a perfusion fluid containing an indifferent colloid and a normal supply of platelet material the rate of oedema is diminished to one-tenth or less of the rate in the absence of platelet material. A further reduction is obtained by adding serum, reducing the rate to from one-third to one-tenth of that in the presence of platelets and indifferent colloid. The oedema with all three such fluids is due to the existence of pores in the capillaries which are permeable to protein. In the frog in the complete absence of platelets, etc., judging from the results of Saslow, the capillary constrictor action of the serum does not much reduce the rate of oedema formation. If these conclusions are true also for mammalian capillaries, it is possible that some cases of oedema are due to absence of platelets, etc., or to impaired activity of the platelets, etc.

The results presented here do not constitute a complete proof that the active material is the platelets. It has merely been shown that activity in preventing oedema is very closely associated with the platelets. However, it has long been supposed that platelets do have an important action in controlling the permeability of capillaries damaged by, for example, an anti-red cell serum, so that it would not be surprising



if the active material is the platelets themselves. This hypothesis will be adopted in the following discussion.

The action of the platelets may be purely mechanical: the flow of fluid through a large pore may draw a platelet across the pore, where it will be held down by the lateral pressure of the blood or perfusion fluid. Platelets will not be held in this way over the pores of the venular capillaries to the same extent, since the flow through those venular capillary pores which are impermeable to protein is in the wrong direction. Also, if at any time a section of capillary is changed, by muscular activity or other cause, to venular from arteriolar function, platelets over the pores will be lifted up by the fluid moving into the capillaries. The reverse process will happen on passing from venular function to arteriolar function; pores will be closed down. It may be that in this way the platelets provide a rather simple automatic mechanism by which the venular capillaries are kept more permeable than arteriolar capillaries, thus promoting the recovery of fluid from the tissues.

The action of the platelets may be to some extent affected by colloid chemical factors.

*Pore size and protein adsorption.* One of the actions of normal serum may be due to protein adsorption on the walls of capillary pores. As Keys [1938] has found, there are ranges of collodion membranes which are permeable to gum acacia in the absence of serum, but perfectly impermeable to acacia in the presence of serum. Similar phenomena are encountered in the ultrafiltration of proteins, and are due to blockage of the pores by adsorbed protein. Addition of substances of superior surface activity, e.g. higher polypeptides, as in Hartley's broth, displaces the adsorbed protein and gum acacia. Such a polypeptide will make a layer on a pore wall not more than  $1.0\text{ m}\mu$  thick. A protein layer will be of the order of 4 or more  $\text{m}\mu$  thick. Thus displacing a protein layer with polypeptide will result in an increase in diameter of  $(2 \times 4 - 2 \times 1) = 6\text{ m}\mu$ , which provides a gap readily permeable to, for example, serum albumin. The increase in rate of oedema development found on adding clupein to serum may be due to this cause, since clupein has a diameter much less than that of serum proteins.

*Relative efficiency of different colloids.* Fig. 7 shows the rate of oedema development with corresponding C.O.P.'s of various colloids. Purified ovalbumin and sheep haemoglobin appear to have the same efficiency, so evidently the colloid permeable pores have a diameter at least equal to that of haemoglobin ( $5\text{ m}\mu$ ). Gum acacia behaves as though only about half of the acacia particles are able to penetrate the pores which

are permeable to haemoglobin. Gum acacia is composed of a number of molecular sizes, with an average molecular weight of 100,000. It thus appears that dilated capillary pores are not readily permeable to molecular weights above 100,000, corresponding to a diameter of 6  $m\mu$ . Saslow's results, however, show that serum albumin at least penetrates fairly freely in the absence of platelets. It thus appears that many of the

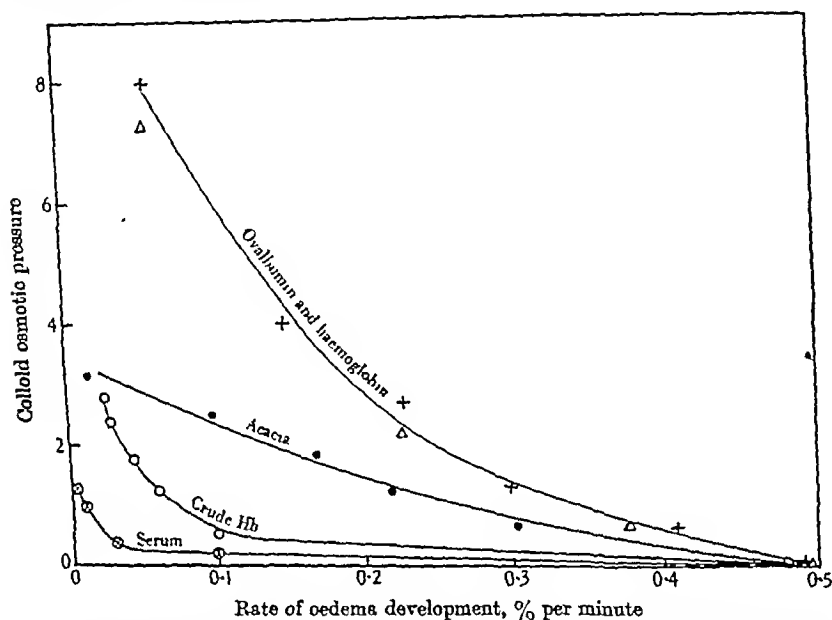


Fig. 6. Rate of oedema with various colloid osmotic pressures of crystallized ovalbumin ( $\Delta$ ), crystallized sheep haemoglobin (+), acacia ( $\bullet$ ), crude ox haemoglobin ( $\odot$ ), ox serum ( $\otimes$ ). The c.o.p. is that given by the formula  $p = C \frac{34,500}{M}$ , where  $C$  is colloid concentration in per cent by weight and  $M$  is colloid molecular weight.

pores of dilated capillaries have an upper limit to their diameter of about 6  $m\mu$ . Contracted capillaries in the presence of platelets are not freely permeable even to ovalbumin, so evidently under such conditions the majority of the pores have a diameter of less than 4  $m\mu$ .

It may perhaps be worthy of note that the variation in the values of  $y$  and  $z$  in equation (6) appears to be systematic. The values of  $y$  are the same (0.14) in all cases fitted by the equation, except that of crude ox haemoglobin (0.067), with which it is probable that the number of available pores is reduced. The values of  $z$  are the same (0.011), except for gum acacia (0.005), for which not all the colloid molecules can pass the pores.

*Relative permeability of skin and muscle capillaries.* When a muscle is perfused with a solution of 2.5 % crude ox haemoglobin, it flushes a delicate pink in the first few minutes as the capillary system is flooded. Then over a period of about 2-4 hr. the colour deepens, as haemoglobin passes into the lymph spaces. It thus takes a maximum of 240 min. for a volume of haemoglobin to penetrate the capillary walls sufficient to fill a volume of about 10 % of the muscle volume, i.e. in the muscle the rate of oedema formation is not less than 0.044 %/min. The average rate of oedema development for the frog as a whole is 0.06 or 0.085 %/min. if tissue only is considered. It thus follows that the capillaries of the skin are possibly more permeable than those of the muscle, but not enormously so.

#### SUMMARY

1. It is shown that, subject to certain assumptions, when a tissue is perfused with an inert colloidal solution the rate of development of oedema should obey the equation  $R = xp^2 - yp + z$ , where  $p$  is the colloid osmotic pressure of the perfusate, and  $x$ ,  $y$  and  $z$  are constants. If the capillaries are not permeable to protein  $R = z' - y'p$ .

2. Solutions of ovalbumin, haemoglobin and gum acacia obey this first equation.

3. Serum, red cells and solutions containing platelet material reduce the rate of development of oedema more than can be accounted for by their colloid osmotic pressure. This activity is not due to the oxygen-carrying capacity of the solutions.

4. Platelet preparations obtained by centrifuging whole blood contain at least 1000 times more material active in preventing oedema than does serum, and 100 times more active material than red cell suspensions prepared by centrifuging and washing red cells.

5. It is suggested that the activity may be due to the platelets themselves, and that the action of the platelets may be largely mechanical, involving simple blockage of protein-permeable pores.

6. Small amounts of clupein added to the perfusate reduce the rate of flow with both acacia and serum. Clupein decreases the rate of oedema with 3½ % gum acacia, but increases the rate with 40 % serum. The action of clupein is at least partially reversible.

I am indebted to the Government Grants Committee of the Royal Society for an apparatus grant.

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## THE INACTIVATION OF ADRENALINE BY PHENOLASES

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THE oxidation of adrenaline by enzymes may occur on the side chain or on the ring structure of the molecule. Oxidation on the side chain is brought about by an enzyme found in tissue extracts [Blaschko, Richter & Schlossmann, 1937 *a, b*]. This enzyme, which is identical with tyraminase [Hare, 1928], is now generally called amine oxidase. The analysis of its action was made possible by the observation that it was not inhibited by cyanide [Blaschko & Schlossmann, 1936 *a*]. Other enzymic systems attack adrenaline because it is a catechol derivative and they are inhibited by cyanide. The experiments described in this paper concern the inactivation of adrenaline by these systems.

From the work of Warburg and Keilin and their collaborators it is known that the enzymic systems which oxidize polyphenols contain either copper or iron, and it is therefore possible to classify them accordingly.

*Catalysts containing copper.* The best studied enzyme of this type is the polyphenol oxidase of potatoes [Kubowitz, 1937, 1938] and of mushrooms [Keilin & Mann, 1938]. Other enzymes which come into this group but have not yet been proved to be copper compounds are the phenolase of the mealworm and of *Sepia officinalis*. Laccase is also an enzyme of this type.

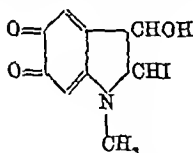
*Catalysts containing iron.* The most important representative of this class is cytochrome-cytochrome oxidase. It oxidizes polyphenols [Keilin & Hartree, 1936, 1938; Green & Richter, 1937], and is the chief respiratory system of all aerobic cells. The oxidation of adrenaline is probably more complex than in the case of polyphenol oxidase, because several stages of oxido-reduction are interposed between adrenaline and oxygen, namely the three cytochromes (*a, b, c*) and the oxidase. Another enzyme of this

group is peroxidase which is also a haematin compound [Kuhn, Hand & Florin, 1931]. It occurs in plants and its chief source is horseradish.

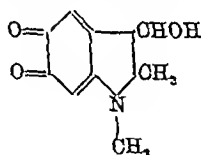
In the previous study on amine oxidase we were able to show that there exists a strict stoichiometrical relationship between inactivation of adrenaline and oxygen uptake. A similar analysis has not hitherto been made for the different cyanide-sensitive enzymes. This has been done in the experiments described in the present paper for polyphenol oxidase, cytochrome-cytochrome oxidase and peroxidase.

There was an additional interest attached to this analysis, since Bacq [1938] claimed that during the oxidation of adrenaline by phenolases a substance with inhibitory properties is formed. This substance he called adrenoxine. Bacq used various enzyme preparations derived from animal and plant tissues, and we have tried to repeat his observations with a similar preparation as well as with the purified enzymes at our disposal.

The oxidation of adrenaline catalysed by phenolases is similar to the oxidation of adrenaline by chemical agents, e.g. iodate, copper or iron salts. These reactions lead to coloured substances which have recently been identified [Richter & Blaschko, 1937; Green & Richter, 1937]. The probable formulae ascribed to the products of the oxidation by iodate (iodochrome of adrenaline) and of the closely related enzymic oxidation (adrenochrome) are given below. Adrenochrome corresponds in its



Iodochrome of adrenaline



Adrenochrome

structure to the red substance formed in the enzymic oxidation of tyrosine, the constitution of which was cleared up by Raper [1927] in his classical studies on tyrosinase.

## METHODS

Adrenaline solutions were incubated with enzyme preparations and the uptake of oxygen was measured manometrically. The initial adrenaline concentration was 1 in 1000. Usually 2 mg. adrenaline dissolved in the equivalent amount of hydrochloric acid in a total volume of 0.2 c.c. was placed in the side bulb of a conical manometer flask; the main flask contained the enzyme and 0.067 *M* sodium phosphate buffer pH 7.3 to bring the total volume to 1.8 c.c. The gas space of the manometers was filled with air, except in the experiments with peroxidase, where no



uptake of oxygen occurs and where the gas space was filled with nitrogen. The flasks were incubated in a bath at room temperature (17–20°). In a single experiment several manometers were set up with the same contents and the uptake of oxygen was measured over suitable intervals. From time to time one of the flasks was removed after a final reading, and the solutions tested at once for their pharmacological activity. It was usually unnecessary to take special precautions to protect the samples from further changes between the final reading and the biological assay, as the oxidation was already slow at the time of removal from the bath. In a few experiments with polyphenol oxidase where the assay was to be done whilst the rapid reaction was still in progress duplicate samples were set up. One was removed and the enzymic reaction was stopped by adding a drop of hydrochloric acid to the sample. At this moment the final reading was taken from the second sample.

In his experiments on the formation of adrenoxine Bacq has used adrenaline solutions 1 in  $10^5$ , and our experiments concerned with the formation of adrenoxine were therefore done not only under the conditions just described but also with solutions (1 in  $10^6$ ), which were kept with the enzyme preparation at room temperature up to 24 hr.

The activity of the samples was assayed on the arterial blood pressure of decerebrate cats as previously described [Blaschko *et al.* 1937*a*], and in some cases of cats under chloralose anaesthesia. A few samples were tested on the isolated rabbit's intestine.

*Enzyme preparations.* The polyphenol oxidase was prepared according to Keilin & Mann [1938]; three preparations were used. One was made from *Psalliota*; it had, when tested on catechol, a  $Q_{O_2}$  of 40,000 (preparation A). The other two were from *Lactarius*; they differed in purity and activity, the  $Q_{O_2}$  of the more active and more purified preparation was 80,000 (preparation B), that of the less active 10,000 (preparation C). For the experiments on the formation of adrenoxine we used in addition press juice from unpeeled potatoes which was prepared on the day preceding the experiment. The cytochrome *c* was a 1 % solution obtained from horse heart according to Keilin & Hartree [1937]; the cytochrome oxidase came from pig's heart [Keilin & Hartree, 1938]. The peroxidase was a preparation from horseradish [Keilin & Mann, 1937]; its activity as measured by its purpurogallin number was 300. It was about 3000 times purified. We are indebted to Prof. D. Keilin, Dr E. F. Hartree and Dr T. Mann for the purified preparations used in this work.

## RESULTS

*Polyphenol oxidase.* Preparation A was used in two series of preliminary experiments in which the inactivation of adrenaline was determined without the uptake of oxygen. The enzyme was used in a 100 and 1000-fold dilution respectively. The initial concentration of adrenaline was 1 in  $10^5$ . Addition of the enzyme to the adrenaline solution led almost instantaneously to the development of a red colour, the maximum redness being reached more quickly with the stronger enzyme solution. When the samples were tested 85 min. after the addition of the enzyme they had no action on the arterial blood pressure of the cat. When retested at different periods during the following  $6\frac{1}{2}$  hr. the samples remained inactive and did not acquire depressor activity. The colour of the solutions had by that time changed from red to yellow-brown and a slight precipitate had formed.

With preparations B and C inactivation and oxygen uptake were measured simultaneously. After the addition of adrenaline to the enzyme solutions an uptake of oxygen occurred which was complete in about 15 min. During this period the reaction was so rapid that the rate of oxidation was probably limited by the diffusion of oxygen into the solution, and our readings therefore give no true measure of the reaction rate. This phase was accompanied by the development of a red colour, the intensity of which increased until the rapid uptake of oxygen had ceased. If the oxygen consumption is expressed in atoms of oxygen per mol. adrenaline it will be seen that in the rapid reaction exactly 2 atoms of oxygen had been used up. After this period a very much slower uptake of oxygen continued for hours and eventually about 6 atoms of oxygen had been taken up. This is illustrated by the experiment of Fig. 1. The manometers were set up as follows:

Main flask: 1.7 c.c. phosphate buffer + 0.1 c.c. preparation B diluted 1:10.

Side bulb: 0.2 c.c. adrenaline hydrochloride (= 2 mg. base).

Inner tube: 0.3 c.c. *N* KOH.

$t = 19.0^\circ$ ; gas phase: air.

The second phase of oxygen uptake was again accompanied by a change in colour. The red colour faded slowly and was replaced by a yellow-brown colour. The assay of the samples on the cat's blood pressure showed that inactivation was complete when 2 atoms of oxygen had been taken up per mol. adrenaline. This is illustrated by Table I, which belongs

to the same experiment as Fig. 1. The amount of oxygen that should be consumed if the inactivation of adrenaline required 2 atoms per mol. is

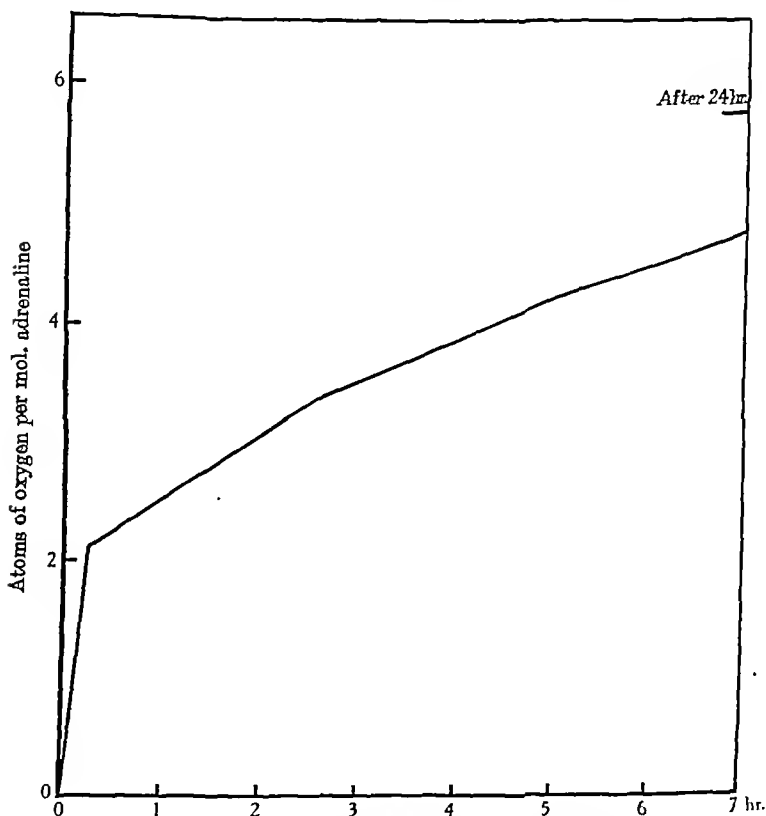


Fig. 1. Oxygen uptake of adrenaline with polyphenol oxidase.

TABLE I. Oxygen uptake and inactivation of adrenaline by polyphenol oxidase

Time of incubation in. min.	Oxygen consumed in c.mm. O <sub>2</sub>	Activity expressed in % of initial activity	
		Calc.	Found
9	130	47	30
10	162	34	30
14.5	210	14	25
31	251	0	0.1
1521	710	0	0

245 c.mm. It can be seen from the data of columns 3 and 4 that inactivation was complete when this amount of oxygen had been taken up. The slight discrepancies between the expected and found values of activity

in the early determinations are of no significance; we have to consider that the oxygen uptake and the inactivation had to be measured in separate samples. When samples had lost their pressor activity and were retested at later stages of oxidation (up to 25 hr.) they never acquired a new pressor or a depressor action. We have carried out such experiments with solutions containing adrenaline in an initial concentration of 1 in  $10^3$  and 1 in  $10^5$  and with the enzyme preparations B and C. These results show that no substance like Bacq's adrenoxine is formed during the oxidation by relatively highly purified preparations of polyphenol oxidase. There was the possibility that the formation of adrenoxine resulted from some impurities contained in the crude preparations used by Bacq. We

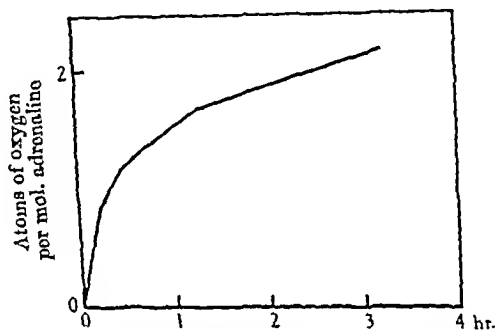


Fig. 2. Oxygen uptake of adrenaline with potato extract.

have therefore repeated the experiments with press juice of potatoes, but, apart from the fact that the oxygen uptake and the inactivation proceeded more slowly, not allowing a sharp differentiation between an initial rapid and delayed slow oxygen uptake, the results did not differ from those previously described. There was no formation of a depressor substance during the oxidation. The assay on the arterial blood pressure had to be made after atropine (1 mg. sulphate intravenously), since the press juice itself caused a fall of pressure on the non-atropinized cat, probably resulting from the presence of a choline ester in the extracts [Oury & Bacq, 1938]. Fig. 2 illustrates the oxygen uptake in an experiment with press juice of potatoes. The flasks were set up as follows:

Main flask: 1.6 c.c. phosphate buffer + 0.2 c.c. potato extract.

Side bulb: 0.2 c.c. adrenaline hydrochloride (= 2 mg. base).

Inner tube: 0.3 c.c. *N* KOH.

$t = 20.0^\circ$ ; gas phase: air.

The curve of oxygen consumption shows a characteristic difference from that in Fig. 1. There is no sharp break in the curve after the uptake of 2 atoms; the rate of oxidation was in fact falling off prior to that point. In agreement with this finding were the results obtained for inactivation. There was no sharp point at which the activity disappeared. In the experiment of Fig. 2, for instance, 5 % of the initial activity was left after an incubation of 18 hr. The only conclusion that can be drawn from this experiment is that the clear-cut action of the purified enzyme is not to be seen, due to interference by other substances in the crude extracts.

*Cytochrome c—cytochrome oxidase.* The oxidation of adrenaline by polyphenol oxidase entails a reduction of the copper in the enzyme which in its turn is oxidized by molecular oxygen. In the oxidation of adrenaline by the cytochrome system there is involved a subsequent oxido-reduction of the three different cytochromes, and probably also of the oxidase before molecular oxygen is brought into reaction. The complexity of this mechanism may be responsible for the initial period of slow oxygen uptake. This period, during which the oxygen uptake slowly increased, was followed by a period of uniform oxidation rate which, however, remained below that observed with polyphenol oxidase. The oxygen uptake slowed down when 2 atoms of oxygen per mol. had been consumed. The break on the curve of Fig. 3 is less sharp than that of Fig. 1. In the experiment from which Fig. 3 is taken the manometer flasks were set up as follows:

Main flask: 1.0 c.c. phosphate buffer + 0.5 c.c. cytochrome *c* solution + 0.3 c.c. cytochrome oxidase.

Side bulb: 0.2 c.c. adrenaline hydrochloride (= 2 mg. base).

Inner tube: 0.3 c.c. *N* KOH.

$t = 19.0^\circ$ ; gas phase: air.

Samples were assayed on the arterial blood pressure of the cat, the results being given in Table II. There was still 4 % adrenaline activity left after about 1 hr. of incubation when approximately 2 atoms of oxygen had been used. This can be understood if we consider that the reaction occurs more slowly than with polyphenol oxidase. A perceptible amount of the first reaction product must already have undergone further oxidation and contributed to the oxygen consumption when some of the adrenaline was still present as such.

Once the pressor activity had disappeared the solution remained inactive even if incubated for a further period of many hours. In no

instance did we observe a depressor activity. Similar results were obtained when the enzyme was incubated with an adrenaline solution 1 in  $10^5$ .

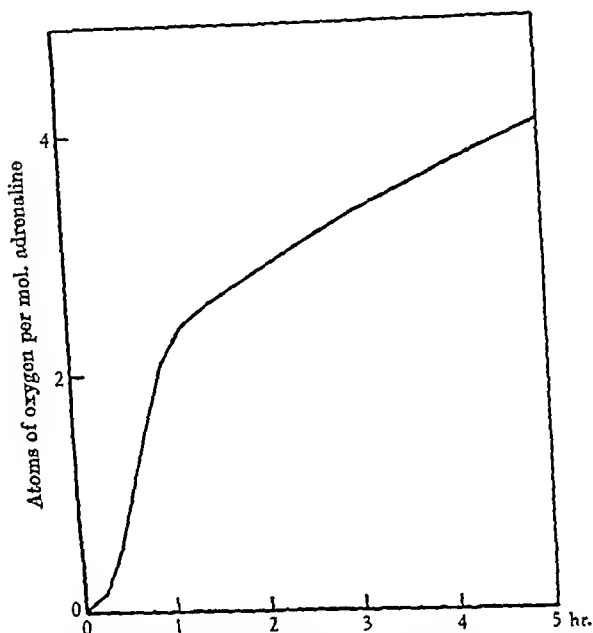


Fig. 3. Oxygen uptake of adrenaline with cytochrome + cytochrome oxidase.

TABLE II. Oxygen uptake and inactivation of adrenaline by cytochrome oxidase + cytochrome

Time of incubation in min.	Oxygen consumed in c.mm. $O_2$	Activity expressed in % of initial activity
51	198	25
63	271	4
175	367	0

*Peroxidase.* Since the oxidation does not involve an uptake of oxygen it cannot be measured manometrically. We have taken the colour changes as an approximate measure for the course of the reaction. As soon as the latter was started by tipping in the adrenaline from the side bulb the solution became pink. The intensity of the colour gradually increased and after 2 hr. the solutions were deep red. Later they became dark brown and a precipitate was formed. With the development of the red colour the solution gradually became inactive and remained so at later

stages when the change to the brown colour occurred. These results are illustrated in Table III, taken from an experiment in which the flasks were set up as follows:

Main flask: 1.6 c.c. phosphate buffer + 0.1 c.c. 0.4 *M* hydrogen peroxide (perhydrol) + 0.1 c.c. peroxidase solution (containing 0.3 mg. peroxidase).

Side bulb: 0.2 c.c. adrenaline hydrochloride (= 2 mg. base).

$t = 17.0^\circ$ ; gas phase:  $N_2$ .

The assay in this experiment was done on the cat's blood pressure and on the isolated intestine of the rabbit. It will be seen that the loss of inhibitor activity on the intestine occurred at about the same time as

TABLE III. Inactivation of adrenaline by peroxidase

Time of incubation in min.	Colour	Activity expressed in % of initial activity	
		Cat's blood pressure	Rabbit's intestine
55	Pink	45	—
95	Red	—	7
135	Red	—	4
165	Red	—	0
191	Brownish	0	—

the loss of pressor activity. When the solution was retested after 3, 4 and 5 hr. on the cat it caused no change in blood pressure. There was no depressor activity. Similar results were obtained in experiments in which the initial adrenaline concentration was 1 in  $10^5$ .

### DISCUSSION

The curves of oxygen uptake in our experiments both with polyphenol oxidase and with the cytochrome system show that two different reactions can be distinguished. There is an initial fast reaction characterized by the development of a red colour, the uptake of 2 atoms of oxygen per mol. adrenaline, and the inactivation of the solution. This is followed by a prolonged reaction during which oxygen uptake is slow and the colour changes to brown. The initial fast reaction is the formation of adrenochrome which requires 2 atoms of oxygen [Green & Richter, 1937]. There is a parallelism between the amount of adrenaline inactivated and the formation of adrenochrome as measured by the oxygen uptake. The second slow reaction is probably not catalysed by the enzymes, adrenochrome being unstable in the presence of oxygen. This reaction does not

lead to new pharmacologically active oxidation products of adrenaline. The formation of adrenochrome by phenolases and its subsequent oxidation is therefore not responsible for the formation of Bacq's adrenoxine.

In the experiments with peroxidase no oxygen uptake could be measured, but the inactivation was also correlated with the formation of a red substance which is very likely adrenochrome. The experiments showed that the excitatory and inhibitory actions of adrenaline disappeared simultaneously. Similar results have been obtained in previous experiments in which adrenaline was incubated with extracts from mammalian liver [Blaschko & Schlossmann, 1936b]. We know now that in these experiments the inactivation of adrenaline must have been brought about mainly by amine oxidase. Thus, oxidation of the catechol group as well as that of the side chain is associated with simultaneous loss of the excitatory and inhibitory properties of the adrenaline molecule.

About the enzymic destruction of adrenaline *in vivo* little is known. Both amine oxidase and the phenolases may be involved. Some of the enzymic systems used in our experiments have not been found in higher animals. Peroxidase has been isolated from plants and polyphenol oxidase from plants and from invertebrates only. Recently, Mann & Keilin [1939] described the occurrence of copper-protein compounds in mammalian tissues. They were not catalytically active, but it is conceivable that similar compounds with phenolase activity exist. The cytochrome system is known to be present in mammalian cells and may participate in the destruction of adrenaline. In fact, the ubiquity of this system in the mammalian organism raises another question, i.e. the protection of adrenaline from the cytochrome system. This protection may be due to substances like ascorbic acid or sulphydryl compounds known to protect adrenaline from inactivation by phenolases *in vitro* [Toscano Rico & Malafaya Baptista, 1935; see also Richter, 1934]. The study of specific inhibitors of enzymes like choline esterase and amine oxidase has opened a new approach to the understanding of pharmacological problems. The inactivation of adrenaline is one of these problems. A recent observation of Clark & Raventós [1939] may find its explanation along these lines. They obtained prolonged action of adrenaline on the frog's auricle after ascorbic acid. Since the heart muscle contains the cytochrome system in high concentration the protection of adrenaline from inactivation by this system might explain the observation of Clark & Raventós.





# THE PERMEABILITY OF THE BLOOD-AQUEOUS HUMOUR BARRIER TO POTASSIUM, SODIUM, AND CHLORIDE IN THE SURVIVING EYE

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THERE is a great deal of evidence that the aqueous humour is a filtrate from the blood plasma, molecules of the size of serum albumen or larger being retained whilst crystalloids are distributed between the two fluids in a manner characteristic of ultra-filtrates *in vitro*. If this is so, then the intra-ocular pressure will be determined by the capillary pressure and the colloid osmotic pressure of the blood; the cause of the raised intra-ocular pressure in glaucoma may be sought, possibly, in a derangement of the normal hydrostatic or osmotic relationships.

Against this view the early idea of Seidel [1920], that the aqueous humour is a secretion, has been revived. Its extreme form has been put forward by Robertson [1939*a*, *b*] and by Robertson & Williams [1939]. They claim that the formation of the fluid is virtually independent of the composition of the plasma and that its pressure is independent of the relative osmotic pressures of the plasma and the aqueous. Such a theory is difficult to reconcile with the classical researches of Henderson & Starling [1904] who showed that the intra-ocular pressure is directly connected with the arterial pressure, and the more recent investigations [Duke-Elder, 1927; Duke-Elder & Duke-Elder, 1931] on the influence of the composition of the blood and of the vascular pressures on the intra-ocular pressure. Recently, Duke-Elder [1938] has put forward the view that the aqueous behaves in a general way as a dialysate, but that its composition and osmotic pressure are modified by an activity of the epithelial cells of the ciliary body. This view promises to reconcile opposing standpoints. The present work is designed to show to what

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extent the membranes lining the living eye can, by a cumulative activity, modify the rate of exchange of ions between the plasma and the aqueous humour, either qualitatively or quantitatively. In this paper we shall refer to the barrier between the blood and the aqueous humour simply as "the membrane"; such a barrier will obviously include such membranes as the endothelial capillary membrane, Bruch's membrane, etc.

Perhaps the only really strong argument made by Robertson in favour of his view is that the membrane of the eye shows uni-directional permeability, crystalloids and water being able to penetrate into the eye but not out of it. Unfortunately this author neither presents experimental evidence nor quotes any reference in support of this claim. If this were so a membrane with this property would, from analogy with other systems, be expected to show secretory functions, although uni-directional permeability *per se* would not necessarily preclude an equilibrium distribution of crystalloids indistinguishable from that in a dialysate. Gaedertz & Wittgenstein [1927], on the basis of experiments carried out chiefly with dyes, claim that the membrane is specifically permeable to anions and impermeable to cations; this fact itself would not justify the postulation of a secretory mechanism, since specific ionic permeability is associated with cells which show no secretory activity, e.g. the erythrocyte [Davson & Danielli, 1938]. The demonstration of a specific anion permeability of the membrane would also provide useful theoretical evidence for secretory activity, whereas a membrane incapable of distinguishing between negative and positive ions would give greater difficulties.

In the present work three main problems have been investigated:

(a) Does the membrane, as claimed by Robertson, show uni-directional permeability to crystalloids?

(b) Is the membrane, as claimed by Gaedertz & Wittgenstein, specifically anion permeable?

(c) Can the membrane secrete ions?

By using the isolated head preparation and varying the concentrations of potassium, sodium and chloride in the perfusing fluid, unequivocal answers to the problems (a) and (b) were obtained, showing that the claims of Robertson and of Gaedertz & Wittgenstein are without foundation. To test (c) a similar preparation was used. Secretion is associated with an oxidative activity of the cells which is normally poisoned by cyanide (see Höber *et al.* [1927-30] in respect of kidney and liver, and Huf [1936] in respect of frog's skin). If, therefore, the cells secrete, then the rate of penetration of an ion, such as potassium, ac

brane, should vary according as the eye is alive or poisoned with cyanide. If this argument is correct, then the results shown here do not indicate that there is a secretion of the potassium ion by the membrane.

The rate of penetration of potassium was measured in the direction from blood to aqueous humour, since it is possible to maintain a reasonably constant high level of potassium in the blood. An attempt was made to measure the rate of penetration of potassium from the eye into the blood. To do this it was necessary to reduce the potassium content of the perfusing blood, and this was done by dialysing the blood against isotonic  $\text{NaCl-NaHCO}_3$ . It was found, however, that the level of potassium in the blood rises so rapidly to its normal value during the perfusion that it was impossible to maintain a concentration difference between the inside and the outside of the eye for long enough to make accurate measurements. Presumably the rise in blood potassium was due to an escape from the muscle [Fenn, 1936] and/or nerve cells.

The rates of penetration of sodium and chloride were measured in the direction of eye to blood, since it is possible to reduce their concentration in the blood by diluting with isotonic solutions containing a colloid, thereby maintaining the relative osmotic pressures reasonably constant. One experiment was carried out with a raised sodium content, i.e. sodium penetrating from blood to aqueous humour, but the result was uncertain, since the large differences in osmotic pressure created by the addition of sodium chloride caused water to pass out of the eye.

### EXPERIMENTAL

The essentials of the procedure were to perfuse an isolated cat's head with cat's blood containing either an excess or deficiency of one of the ions, potassium, sodium or chloride. The aqueous of one eye was used to obtain the initial value of the concentration of the ion considered, and after a definite interval of time the aqueous of the other eye was withdrawn for analysis. Determinations of the concentration of the ion in the blood serum during the perfusion gave the concentration difference between the serum and the aqueous, and from these values a permeability constant could be calculated.

When it was desired to compare the rates of penetration of a given ion into the living and dead eyes of the same head, a determination of the concentration of the ion in the animal's own blood at the time of severance of the head enabled us to make an approximate calculation of the concentration initially present in the aqueous. Consequently the with-

drawal of the aqueous from the one eye was made after 1 hr. of perfusion, and from the other after a further hour during which the head was poisoned with cyanide. In this way two permeability constants were obtained, one for the penetration into the living eye, and one into the poisoned eye.

*Details of perfusion.* The cat was anaesthetized with ether and subsequently with chloralose. The dorsum of the second cervical vertebra was exposed and cleaned, and a dissection was then made to expose the common carotids for about  $1\frac{1}{2}$  in. to their bifurcation. Portions of the larynx and trachea were removed. The common carotids were clipped, cannulated and connected with the perfusion circuit. Just before the occlusion of the vertebral circulation with an *écraseur* the clips on the carotids were removed, thereby establishing the perfusion of the head. As a result the head was not deprived of a fully oxygenated blood supply at any time during the procedure. A Dale-Schuster pump was used to propel the blood through the perfusion circuit which included a resistance and an oxygenator, the latter being of the type used by Gregory [1939] and Chute & Smyth [1939]. A current of 95%  $O_2$  and 5%  $CO_2$  was passed through it. The temperature of the circulating blood was maintained at 37° C. by immersing most of the circuit in a water-bath, the temperature of which was controlled by a thermoregulator of the type used by Lythgoe & Quilliam [1938]. Two cats were usually bled under ether anaesthesia to provide defibrinated blood for the perfusion apparatus before the experiment. For further details of the procedure the reader is referred to a paper by Chute & Smyth [1939]. The perfusion pressure was maintained as constant as possible at 180 mm. Hg. The flow varied with different animals between 40 and 90 ml./min. and invariably increased after poisoning the preparation with cyanide.

The isolated head thus prepared exhibits a blink reflex in response to a puff in the eye, or to a movement of the vibrissae, or to a direct stimulation of the cornea or the inner canthus of the eye. A tap on the nose elicited a jaw jerk and a blink, and often in raised blood potassium experiments a series of jaw movements. The pupil of the eye was constricted, and spontaneous eye, ear and jaw movements were occasionally seen. These reflex movements were invariably more marked in preparations perfused with blood of high potassium content. When the isolated head is deprived of an oxygenated blood supply, or is poisoned with cyanide, the reflexes vanish and the pupils dilate widely. In the dying head, gasping movements of the jaws and a series of movements of the alae of the nostrils were observed. The constriction of the pupil was taken

as an indication that the preparation was alive. Eye fluids for analysis were withdrawn with a clean dry syringe.

*Chemical methods of analysis.* Davson [1939] has recently described an exceptionally accurate method for the determination of sodium in serum and aqueous humour involving the Barber-Kolthoff [1928] gravimetric precipitation. This method was used in this work for both sodium and potassium, the latter being precipitated by the Kramer [1920] sodium cobaltinitrite procedure and estimated volumetrically. The chloride was determined by the method of Sendroy [1937].

### THEORETICAL

The rate of penetration of a substance into the eye will be given by the following equation:

$$\frac{dx}{dt} = kA (C_s - C_{aq});$$

$x$  is the amount of the substance penetrating into the aqueous humour,  $C_s$  and  $C_{aq}$  are the concentrations of the substance in the serum and aqueous humour respectively,  $A$  is the area of the membrane through which diffusion occurs,  $k$  is a permeability constant, and  $t$  is the time in min.

This assumes that the rate of passage across the membrane is slow compared with the rate of diffusion in the eye and blood.

Since the concentration of the substance at any moment is given by  $C_{eq} = (x + I)/V$ , where  $I$  is the amount initially present in the eye and  $V$  is the volume of the fluid in the eye, which remains virtually constant, we get

$$\frac{dC_{aq}}{dt} = \frac{kA}{V} (C_s - C_{aq}),$$

which gives

$$\begin{aligned} \frac{1}{(t_2 - t_1)} \log \frac{(C_s - C_{aq})_{t_1}}{(C_s - C_{aq})_{t_2}} \\ = \frac{kA}{V} \frac{1}{2.303} \\ = K, \end{aligned}$$

assuming that the serum concentration remains constant (actually the serum concentrations did vary slightly so that a mean value for the whole period was used),  $t_1$  is the time at the beginning and  $t_2$  the time at the end of the experimental period.

Thus if it is assumed that  $A/V$ , the ratio of the area of the blood-aqueous barrier to the volume of the eye is constant for different eyes, the logarithmic ratio may be considered as a measure for comparison of the rates of penetration of a given substance into the eye. So that the

greater the rate of penetration, the greater will be the value of  $K$ . Such a treatment is not strictly correct for ionic permeability, since potential differences will be set up owing to the unequal rates of diffusion of the positive and negative ions. Since many of the basic assumptions of the equation are first approximations only, and further, since the experiments show that the relative rates of penetration of anions and cations are not greatly different, a more exact treatment would be supererogatory. It should be noted that  $K$ , the measure of the rate of penetration of any given ion, is independent of the concentration units used so that for the convenience of appreciating the relative magnitude of the changes in concentration, the actual concentrations have been scaled so that  $A_1$ , the initial concentration in the aqueous humour, is equal to 100.

### RESULTS

In Table I the initial concentration of the potassium ion in the aqueous humour ( $A_1$ ) is made equal to 100, and its concentration in the aqueous humour after a definite interval of perfusion ( $A_2$ ) and its mean value in the serum ( $S$ ) are scaled up appropriately.

Inspection of the values of  $K$ , the measure of the rate of penetration of potassium, shows a variability between the extremes of 17 and 36, with a mean value of 24 for the rate of penetration into the living eye,

TABLE I. Penetration of potassium into the aqueous humour. The concentrations of potassium are scaled so that  $A_1$ , the initial aqueous concentration, is made equal to 100.  $A_2$  is the aqueous concentration after perfusion with a serum potassium of  $S$ . The time period in all cases was 1 hr. except in the experiment 12. i. 39, where it was 75 min.

Exp.	$A_1$	$A_2$	$S$	$100 \left\{ K = \frac{1}{t_2 - t_1} \log \frac{(S - A_1)t_1}{(S - A_2)t_2} \right\}$	Remarks
3. i. 39	100	134	160	36	Alive
21. xii. 38	100	163	256	23	Alive
14. xii. 38	100	148	253	16	Alive
7. xii. 38	100	155	220	27	Alive
15. xi. 38	100	115	143	20	Alive
18. i. 39	100	160	227	28	Alive
5. v. 39	100	121.5	149.5	25	Alive
11. v. 39	100	122.5	160	20	Alive
15. v. 39	100	123.5	174	17	Alive
				Mean = 24	
12. i. 39	100	176	258	23	Poisoned
24. iii. 39	100	124.5	166	20	Poisoned
31. iii. 39	100	134	195	19	Poisoned
18. v. 39	100	147	206	25	Poisoned
25. v. 39	100	138.5	185	26	Poisoned
28. vi. 39	100	116	146	19	Poisoned
				Mean = 22	

Mean value of  $K$  for all experiments on live and poisoned heads = 23.

against a mean value of 22 for the poisoned eye. In view of the individual variations a difference between the means of only two units is without significance. Thus, so far as these experiments go, there is no great difference in behaviour between a living and a dead eye in respect to the rate of penetration of potassium. Whether or not the variability of the preparations is due to different permeabilities of the membrane, or merely to variations in  $A/V$ , cannot be decided at present. The fact that, out of the nine experiments on living eyes, six values of  $K$  differ by not more than four units from the mean, indicates a regularity in behaviour in response to a raised blood potassium that is reconcilable with a mechanical diffusion process.

In an endeavour to obtain more accurate evidence as to the possible difference in behaviour of living and dead eyes, experiments were carried out in which the head was perfused with a blood containing a raised potassium content for two successive hours, 1 hr. alive and 1 hr. either alive or poisoned with cyanide. In this way a direct comparison on the same animal could be made, provided a value for the initial concentration in the aqueous humours of the eyes before the perfusion began could be obtained without withdrawing the aqueous humour. An approximate value can be obtained by estimating the potassium content of the serum of the animal whose head was used in the experiment, and multiplying by the factor  $1/1.07$ , which is the average ratio of the concentrations of potassium in the aqueous humour and serum at the end of the operative procedure. The high value is due to a gradual increase in the potassium content of the blood under anaesthesia, the concentration in the aqueous humour lagging behind. This unfortunately may introduce a rather large error into the calculation, perhaps in an extreme instance of approximately six units. It is not sufficient, however, to mask any large change in permeability which would be expected if cyanide suppresses a secretory mechanism. The results of this series of experiments are shown in Table II, where " $A_1$ " is the calculated value of the initial concentration of potassium in the aqueous humour,  $A_2$  is the value after 1 hr. of perfusion and  $A_3$  after a further hour.  $S_1$  and  $S_2$  are the mean values of the serum potassium concentration during the first and second hours respectively.  $K_1$  and  $K_2$  refer to the values of

$$\frac{1}{t_2 - t_1} \log \frac{(S - A_1)_{t_2}}{(S - A_2)_{t_2}}$$

calculated for penetration during the first and second hours.

In the first three experiments shown in Table II, the head was alive for both periods of 1 hr., and it is seen that the values of  $K_1$  and  $K_2$  are



TABLE II. Penetration of potassium into the aqueous humour of both eyes. The concentrations of potassium are scaled so that " $A_1$ ", the calculated initial aqueous concentration before the perfusion, is made equal to 100.  $A_2$  and  $A_3$  are the observed aqueous concentrations after 1 and 2 hours' perfusion with a blood containing potassium in the mean concentrations of  $S_1$  and  $S_2$  respectively.

Exp.	" $A_1$ "	$A_2$	$A_3$	$S_1$	$S_2$	$K_1 \times 100$	$K_2 \times 100$	Remarks
15. v. 39	100	144.5	179	244	252	16	17	Alive
11. v. 39	100	161	197	258	252	22	20	Alive
5. v. 39	100	151	184	226	226	23	25	Alive
25. v. 39	100	171	237	298	317	19	26	Poisoned
31. iii. 39	100	140	188	248	275	14	19	Poisoned
24. iii. 39	100	140	174	206	233	21	20	Poisoned
28. vi. 39	100	134.5	156	187	196.5	22	19	Poisoned

reasonably constant. In the last four experiments the head was poisoned with cyanide at the beginning of the second hour, and it is seen that although there is a slightly greater variation in the relative values of  $K_1$  and  $K_2$  the differences in any given experiment fall within the limits of experimental error. A slight increase in the value of  $K$  during the second hour might be expected owing to the increased flow that occurs after poisoning with cyanide.

In Table III are shown some results on the diffusion of sodium and chloride.

TABLE III. Permeability of the aqueous humour-blood barrier to sodium and chloride. The concentrations are scaled so that  $A_1$ , the initial aqueous concentration, is made equal to 100.  $A_2$  is the aqueous concentration after perfusion for one hour with a serum concentration of  $S$ . In experiment 25. i. 39 penetration is in the direction blood to aqueous humour, in the remaining experiments the direction is from aqueous humour to blood.

Exp.	$A_1$	$A_2$	$S$	$K \times 100$	Remarks
Sodium					
17. iii. 39	100	94	75.5	10	Alive
22. ii. 39	100	90.5	70	17	Alive
19. v. 39	100	97.5	74.5	5	Poisoned
26. v. 39	100	96.5	80	8	Poisoned
31. v. 39	100	94.5	60	6	Poisoned
				Mean = 9	
25. i. 39	100	111	119	38	Alive
Chloride					
19. vi. 39	100	94.5	80	14	Poisoned
14. vi. 39	100	94	78	14	Alive

In the first five sodium experiments the concentration of sodium in the serum was reduced by the addition of a diluting mixture consisting of isotonic glucose and gelatine, or gum arabic; in this way the osmotic pressure was maintained as constant as possible. It is very difficult to make viable preparations using such a perfusion fluid, and a number died soon after the change over. When this occurred, cyanide was added

to ensure complete suppression of any secretory activity and the values of  $K$  so obtained are presented in Table III. It should be emphasized here that we do not wish to make a close comparison between the permeabilities of living and dead eyes to sodium and chloride owing to the small number of experiments on these ions. Our main desire is to show that sodium and chloride can diffuse out of the eye. If the suppression of a secretory activity could be expected to produce a marked alteration in the permeability to sodium and chloride then of course a comparison of the figures presented would certainly indicate the absence of a secretory activity. The results, however, show beyond doubt that sodium can penetrate the membrane between the blood and the aqueous humour. In the sixth experiment the sodium content of the blood was raised by the addition of  $M$  NaCl. This increased the osmotic pressure and therefore caused a loss of water from the eye; this was apparent by the marked decrease in the intra-ocular pressure<sup>1</sup> as soon as the sodium chloride was added. It is to be noted that the value of  $K$  in this instance is 38 against values of 5-17 for the rate of penetration in the opposite direction, and this difference is doubtless due to the migration of water producing an apparent penetration of sodium. Whether any sodium actually penetrates cannot be proved.

In the same table two experiments are shown on the rate of loss of chloride from the eye, one on a live head and the other on a poisoned one. In these cases the blood was diluted with isotonic  $\text{NaNO}_3\text{-NaHCO}_3$  solution. The results show no difference in behaviour between the living and dead eye. The close agreement between the two values is accidental. The point that we wish to emphasize from the results in Table III is that sodium and chloride are able to migrate across the membrane.

The mean value of  $K$  for the penetration of sodium from the aqueous humour to the blood is 9 compared with a value of 23 for potassium penetrating in the reverse direction. This difference is significant, and is what one would expect from the comparative sizes of the two ions. Chloride seems to diffuse at about the same rate as sodium, thereby showing that Gaedertz and Wittgenstein's claim of specific anionic permeability is both qualitatively and quantitatively unfounded.

<sup>1</sup> This observation confirms the earlier work of Duke-Elder [1927], showing that the intra-ocular pressure is an inverse function of the osmotic pressure of the blood. It may also be noted that reduction of the colloid osmotic pressure of the blood by diluting it with isotonic sodium chloride solution causes a large increase in intra-ocular pressure. Consequently Robertson's [1938] claim, based on measurements involving the use of the inaccurate tonometer, that a reduced colloid osmotic pressure has no influence on the intra-ocular pressure is not borne out by experiments on the surviving eye.

## DISCUSSION

The results described in this paper prove that potassium and sodium may penetrate into the eye and that sodium and chloride may pass out; consequently, any secretory theory of the formation of the aqueous humour, which assumes an irreciprocal permeability of the membrane or a specific ionic permeability, is without foundation.

It should be noted that the experiments of Gaedertz & Wittgenstein, which led them to conclude that the membrane is specifically permeable to anions, were based essentially upon studies of the penetration of dyestuffs into the eye. Acid dyestuffs were found to penetrate, whereas basic dyes, e.g. neutral red, did not. Most dyestuffs are weakly acidic or weakly basic, and it is now generally accepted that the mechanism of penetration of salts of weak acids and bases through a membrane is essentially different from that of salts of strong acids and bases, such as NaCl; the former being brought about by the penetration of undissociated acid or base produced by hydrolysis [see Tyler & Horowitz, 1937; Kralh & Clowes, 1938], followed later by ionic exchanges; the latter by ionic exchanges alone. Thus salts of the weak base ammonium penetrate readily into the erythrocyte, which is impermeable to cations, the mechanism being most probably the initial penetration of ammonia followed by an exchange of anions [Jacobs, 1927; Jacobs & Parpart, 1938]. The demonstration of the rapid penetration of salts of weak bases such as ammonium into the erythrocyte does not prove that its membrane is specifically cation permeable, but rather supports the opposite view that it is specifically anion permeable.

There are many other objections to the use of dyes in permeability studies which need not be entered into here. It is enough to say that the apparent failure of a dye to penetrate into a given cell is not only due to an impermeability of the cell membrane to molecules of this dye, but involves the problem of vital staining, a much more complicated subject which has been exhaustively reviewed by Gicklhorn [1931].

An interesting investigation based on the use of dyes was made by Friedenwald & Stiehler [1938]. They claim that the epithelium of the ciliary body shows a selective permeability in that water and basic dyes are preferentially transferred in the direction of blood to aqueous humour, and acidic dyes in the reverse direction. This selectivity is said to be due to the concentration of Warberg's yellow enzyme in the ciliary epithelial cells. In view of the fallacy in arguing from the behaviour of dyestuffs to the behaviour of ions in general, it is perhaps unfortunate

that these striking claims rely essentially on observations of selective staining. In addition Rose Bengal which was principally used in this investigation is a strong haemolytic agent in concentrations above  $1 \times 10^{-6} M$  in the presence of light and above about  $1 \times 10^{-4} M$  in the dark [Blum, Pace & Garrett, 1937]. Friedenwald & Stiehler used a concentration of  $1 \times 10^{-3} M$ . The action of lysins of this class is not confined to the erythrocyte [Lillie, Hinrichs & Kosman, 1935].

Proponents of the secretory theory argue that the distribution of diffusible substances between the aqueous humour and the plasma is not in accordance with that required by the Donnan equilibrium. The evidence from chemical analyses is certainly conflicting. To quote only the more recent publications, Davson, Duke-Elder & Benham [1936] find that the sodium, potassium and chloride ions are distributed approximately in accordance with theory, whereas Hodgson [1938] finds the excess of chloride in the aqueous humour to be too great. More recently, Davson [1939] has carried out analyses of sodium in the aqueous humour and the plasma of cats, avoiding disturbances due to anaesthesia and using a method which gave a mean error of about 1 in 500. He found that although individual cats showed variations in their distributions from a theoretical ratio of 1.04, the variations were no larger than those in experimental ultra-filtrates and dialysates. Similarly, Stary & Winternitz [1932] showed that although the concentration of calcium in the aqueous humour is 7.4 mg.% compared with a value of 12.1 mg.% in serum, the concentration in an ultra-filtrate of serum was 7.4 mg.%, i.e. the same as that in the aqueous humour. The explanation of the discrepancy from the theoretical value is that some of the calcium in serum is indiffusible. Deductions from estimations of the relative concentrations of non-electrolytes in serum and aqueous humour present some difficulty, since (a) the chemical methods may not be sufficiently specific, so that comparison between a protein-free and a protein-rich fluid is not justified, and (b) where the non-electrolyte is a metabolite, its concentration in the aqueous humour and plasma will vary continuously. The results of Walker [1933] and Adler [1933] on non-electrolyte distribution conflict with the dialysis theory. It would not be fair to dismiss them on the above grounds alone. The decision as to whether there is an active secretion of urea, glucose, etc., will depend on investigations of the sort described here. Nevertheless, it would be very unsafe to argue that, because some non-electrolytes are not equally distributed between the two fluids, there is therefore an active secretion of these substances, either into or out of the eye. This point is well exemplified by the work of

Fischer [1930], who found that the distribution of lactic acid between blood and aqueous humour was different in normal and aphakic eyes, the concentration in the aqueous in the former being larger, presumably owing to the continuous addition of metabolites from the lens which would be absent in the aphakic eye.

Attention should be drawn to the recent observation of Benham, Duke-Elder & Hodgson [1938] that the osmotic pressure of the aqueous humour is higher than that of the blood; i.e. the osmotic pressure relations are the opposite to those required by the Donnan equilibrium. The method of measurement depended on the relative rates of evaporation of two fluids [Hill, 1930], and there is a possibility that the proteins in serum are capable of modifying the rate of evaporation without modifying the osmotic activities of the other molecules in serum [Rideal, personal communication]. Until such an acceleration in the rate of evaporation can be proved the results just quoted present a stumbling block to the acceptance of a simple ultra-filtration mechanism for the formation of the aqueous humour.

A novel argument in favour of a secretory mechanism is that of Robertson [1939*a*]. This author has followed the penetration of glucose, urea, etc., into the lymph, the gastric juice, the cerebrospinal fluid and the aqueous humour after an intravenous injection of the substances. He argues that, because the course of penetration into the aqueous humour follows that into the gastric juice more closely than that into the lymph, the aqueous humour is a secretion. Reference to the equation derived to suit the conditions of penetration into the eye will show that the rate of variation of the concentration of the penetrating substance at any given moment is a function of  $A/V$ , the ratio of the area of the membrane to the volume of fluid in the eye. For comparison between rates of penetration into two different systems, this ratio must be known, otherwise differences in the rate may simply be attributable to differences in this ratio and not to any special characteristic of the membrane. It is clear that in two such anatomically different systems as the optic and lymphatic even an approximate equality of  $A/V$  is unlikely, so that the comparisons are without value. Quite apart from this consideration the argument ignores certain obvious dissimilarities of the circulation of the fluids compared.

This brief review of the problem of the origin and conditions of maintenance of the aqueous humour shows that there are many points to be settled. Clearly, then, a decisive answer to the question "Is the aqueous humour a dialysate?" cannot be given until

work is forthcoming. The results described in this paper, however, indicate that the influence of a secretory activity in respect to the potassium, sodium and chloride ions is negligible.

### SUMMARY

1. The surviving isolated head was perfused from a pump oxygenator circuit, the concentrations of the potassium, sodium and chloride ions in the perfusing fluid were varied and the rate of penetration of the ions into the aqueous humour was determined chemically.

2. In response to a raised potassium content of the perfusing fluid potassium penetrated into the eye; the rate varied from animal to animal, but eleven out of fifteen experiments gave values of a "permeability constant" ( $K$ ) not differing from the mean by more than 16%. The mean ( $K$ ) of a group of experiments on living eyes was not significantly different from that of a group of eyes poisoned with cyanide.

3. In response to a decreased sodium content of the perfusing fluid sodium penetrates from the eye into the blood; the rate of penetration is about one-third of the rate for potassium.

4. In response to a decreased chloride content of the perfusing fluid chloride diffuses out of the eye; the rate is about the same as that of sodium.

5. The results show that the membrane separating the eye fluids from the blood is not specifically impermeable to cations, as claimed by Gaedertz & Wittgenstein, nor does it show unidirectional permeability as claimed by Robertson. The experiments comparing the behaviour of living and poisoned eyes indicate that the membrane does not secrete potassium and probably not sodium or chloride.

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A.C. produces a twitch as well as a tonic contraction; these two contractions are antagonistic. The tonic contraction is characterized by slow relaxation, and is produced by ions outside the muscle fibres, as it is produced if stimulating ions are added to the external solution. A process (resulting in a twitch) produced by A.C., and probably due to the mobilization of ions within the muscle fibres, is antagonistic to the action of ions without the fibres, so that when the A.C. is stopped, the disappearance of this process brings into effect the action of ions outside the fibres which had been antagonized during the passage of A.C.; this results in an A.C. off-contraction.

The above experiment suggests that the passage of the current affects ions both inside and outside the muscle fibres; the result depends upon the sensitivity of the muscle to ions outside. If the muscle is less sensitive, then a twitch is produced. If the sensitivity is greater, the twitch decreases but the relaxation is prolonged; if the sensitivity is great, as in cyanide with *Mytilus* muscle, the twitch disappears and a tonic contraction is produced. Veratrine antagonizes tone as well as the tonic contraction produced by A.C.

(4) The sodium chloride content of mammalian saline is greater than that of frog saline, so that mammalian plain muscle withstands the presence of abnormal substances better than frog muscle, but is much inferior in this respect when compared to *Mytilus* muscle.

*Similarities between the responses of mammalian and frog or Mytilus muscles.* Both muscles exhibit two kinds of contraction, tonic and phasic. When the muscle is in great tone, or tonic contraction is induced by other substances, such as drugs or anions, the excitability diminishes. Hence tonic contraction is one of the factors that affects the two excitabilities similarly; this is presumably due to increased viscosity. Correspondingly, substances that diminish tone, such as small concentrations of drugs, or ions, increase the excitability. During a contraction produced by A.C. potassium, or acetylcholine, tone diminishes, so that a twitch is antagonistic to tone. As most agencies affect the excitability to A.C. and potassium similarly, it was not easy to define the existence of two excitabilities in mammalian muscle; the following reactions, however, demonstrate these.

Increase in the concentration of hydrogen ions from pH 6.5 to 5 diminishes the excitability to A.C., but increases that to potassium. Increase in the concentration of calcium from 0.002 *M* to 0.03–0.06 *M*  $\text{CaCl}_2$  decreases the excitability to A.C., but increases that to potassium. This is due to the fact that owing to slow adaptation, hydrogen and

This is the most important difference between the responses of frog and mammalian unstriated muscle, and accounts for most of the other differences between the reactions of the two muscles. These are:

(1) The majority of the substances used, anions, cations and drugs, affect the excitability to A.C. and potassium in the same direction; in mammalian muscle therefore that factor which affects the excitabilities similarly is predominant. This is to be expected, as adaptation appears to play a prominent part in determining the response of the muscle.

(2) Mammalian muscle is sensitive both to electrical and chemical stimulation owing to slow adaptation. Contraction is caused by small concentrations of drugs and ions. Dog stomach reacts to adrenaline, hence the inexcitability of frog stomach to adrenaline is due to rapid adaptation.

(3) The A.C. off contracture is absent in mammalian muscle, and so also there is no hyperexcitability to chemical stimulation at the end of a prolonged period of stimulation with A.C. This is interesting, as it shows that the A.C. off contracture is associated with the integral process of excitation resulting from A.C. stimulation, and not to extraneous factors, such as electrolysis of the saline. It is associated with rapidity of adaptation.

The properties of the A.C. off-contracture are those of the potassium contraction. This shows that, during stimulation with A.C., the ions place themselves in a position similar to that when they are added from outside—that is outside the muscle fibres. The absence of the A.C. off-contracture in mammalian muscle, which is sensitive to chemical stimulation, shows that it is not due to ions in the solution. There appears to be a transference of ions from the interior to the exterior of the muscle fibres; one kind of adaptation is associated with this phenomenon and affects the two excitabilities in opposite directions. The other kind of adaptation is produced by calcium and affects the two excitabilities similarly. The A.C. off-contracture is associated with the restitution of the ions into their original position. The above view is further supported by the fact that in *Mytilus* muscle, the A.C. off-contracture increases in the absence of calcium, which is known to diminish the permeability of membranes.

There is, however, another kind of A.C. off-contracture which is associated with decreased adaptation, such as in cyanide or increasing temperatures (20–30° C.). This is probably due to the action of ions in the solution, and reinforces the first A.C. off-contracture. The mechanism of this contracture is explained by the following observation.

about pH 9 to 7; thereafter they both increase (experimental range to pH 4). Hydrogen ions in small concentrations are inhibitory; in larger concentrations they are stimulatory and potentiate the response to potassium.

The optimum temperature for A.C. is about 23–24° C., as found by Winton [1926]. Tone also increases up to 23° C., it then declines to about 30° C. and increases again up to 45° C. It is interesting to note that the temperature of 37° C. is more conducive for tone, spontaneous activity, and chemical stimulation, than for response to A.C. stimulation.

### *Withdrawal contractions*

A most significant feature of mammalian plain muscle is the contraction produced by withdrawal of certain substances, ammonium (6 out of 6 muscles), nitrate (6 out of 6 muscles), thiocyanate (6 out of 6 muscles, Fig. 2), adrenaline (2 out of 7 muscles, Fig. 3). As judged by the effect of hydrogen and calcium ions, the former three contractions belong to the potassium group, that is, they are produced by ions outside. Their withdrawal sensitizes the muscle to such ions. The contraction produced by withdrawal of adrenaline has not been classified, owing to the uncertainty of its production.

A characteristic action of these substances is that they at first cause relaxation of the muscle. Their withdrawal produces an opposite change. That this withdrawal contraction is associated with this initial relaxation is shown by the facts that (1) in frog sartorius only subliminal concentrations of thiocyanate produce contraction [Gelhorn, 1931]; (2) in *Mytilus* muscle withdrawal of adrenaline (1 in 50,000), and acetylcholine (1 in 50,000) may produce a contraction in insensitive muscles, which relax instead of contract on the introduction of these substances, i.e. they decrease tone, and when withdrawn, produce an opposite change, viz. increase in tone; (3) it does not occur with those substances that do not produce this initial relaxation. Just as withdrawal of inhibitory substances causes contraction, moreover, withdrawal of stimulating substances such as potassium, acetylcholine and cessation of A.C. (10 V.–10 sec.) produces relaxation.

The above phenomena are significant, as they show that the muscle adapts to the action of ions outside, whether inhibitory or stimulatory, by producing an antagonistic effect. This may be related to a change in calcium concentration, which antagonizes the action of univalent ions, inhibitory or stimulatory.



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Withdrawal of sodium chloride by replacement with lithium chloride or sucrose produces a contraction; the contraction produced by hypotonic saline is mainly due to the withdrawal of sodium chloride.

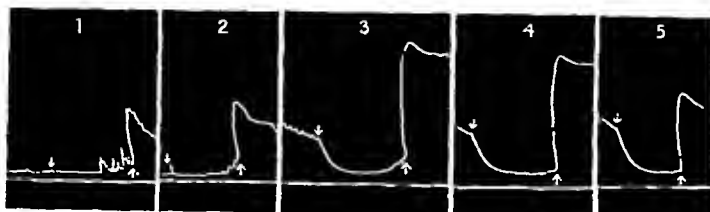


Fig. 2. Contractions produced by withdrawal of thiocyanate (Cl of the saline replaced with SCN). Added at ↓ and withdrawn at ↑. Period of immersion in SCN, 10 min. 1st and 2nd contractions in normal saline; 3rd in 0.01 *M* CaCl<sub>2</sub>; 4th in 0.02 *M* CaCl<sub>2</sub>; 5th in normal saline again. In 1st and 2nd figures, there is no initial relaxation, as the muscle is already relaxed; in others there is initial relaxation. 0.01–0.02 *M* CaCl<sub>2</sub> potentiates the response to potassium and antagonizes that to A.C., so that the SCN withdrawal contraction belongs to the former group.

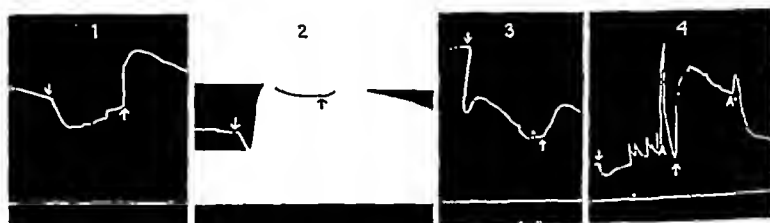


Fig. 3. Withdrawal contractions. As in Fig. 2. 1st contraction is produced by withdrawal of NaNO<sub>3</sub>, 2nd by withdrawal of ammonium. Note that ammonium at first causes relaxation, then contraction; the contraction on withdrawal of ammonium is not marked as the muscle is already contracted. 3rd figure shows the effect of 1 in 10<sup>5</sup> adrenaline; there is marked initial relaxation followed by contraction and then relaxation. 4th figure shows the effect of 1 in 10<sup>5</sup> adrenaline, the muscle being stimulated with A.C. 15 V.–10 sec. On the addition of adrenaline there is relaxation followed by contraction, the muscle is stimulated at A with A.C. On withdrawal of adrenaline there is a tonic contraction antagonistic to A.C. (cf. the effect of A.C. on D.C. contraction in *Mytilus* muscle). Similarly withdrawal of anions is antagonistic to A.C.

Ammonium and potassium (0.02 *M* KCl in *Mytilus* and frog muscles and larger concentration in mammalian muscle) have an action opposite to that of sodium chloride.

An ammonium withdrawal contraction, having the properties of the A.C. contraction as in frog muscle, has not been observed in mammalian muscle. Increase in osmotic pressure produces a contraction, but the restoration of original osmotic pressure produces a further contraction.

## DISCUSSION

There are two views which attempt to explain the mode of action of substances in producing excitation. Straub [1902] holds that a difference in concentration of ions within and without the cells produces excitation, while Clark [1933] thinks that ions affect the surface by attaching themselves to sensitive patches. To the author both these views appear correct.

Two factors affect the excitability of plain muscle [Singh, 1939f]; one of these is the difference in concentration of ions within and without the fibres, and the other is probably the surface membrane. Facts which suggest that differences in concentration of ions within and without the fibres cause excitation have been mentioned previously. That changes in a surface factor also produce excitation is suggested by the following observations: (1) In *Mytilus* muscle small concentrations of calcium cause relaxation; (2) very small concentrations of drugs (1 in  $10^6$ ) produce changes in excitability, (3) sudden stretch or release causes excitation; this will produce sudden changes in the surface. (4) touch, such as by fingers or bubbling, causes contraction of plain muscle; these obviously can primarily affect the surface only. The existence of two factors is clearly shown by the effects of calcium or potassium on frog or *Mytilus* muscles. Small concentrations increase the excitability to A.C. as well as to potassium. Larger concentrations cause contraction, and are antagonistic to A.C., the effect of larger concentrations is antagonized by increase of osmotic pressure. It appears that a difference in concentration of ions within and without the fibres affects the surface indirectly in some way or vice versa.

## SUMMARY

1. Compared to frog plain muscle, adaptation in mammalian plain muscle is slow, this enables the latter to function at higher temperatures at which the excitability of frog muscle is low, as adaptation in frog as well as mammalian muscle increases with temperature.

- 2 The majority of the substances, anions, cations and drugs, affect the excitability to A.C. and potassium in the same direction, but the two excitabilities can be distinguished by the differential effect of hydrogen and calcium ions.

3. Mammalian plain muscle is highly sensitive both to electrical and chemical stimulation owing to slow adaptation.

4. Mammalian plain muscle does not exhibit the A.C. off-contraction.



5. Marked changes in excitability are produced by very small concentrations of drugs.

6. Contraction is caused by withdrawal of nitrate, thiocyanate and ammonium ions.

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## DISCHARGES FROM THE SENSORY ORGANS OF THE CAT'S VIBRISSAE AND THE MODIFICATION IN THEIR ACTIVITY BY IONS

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RECORDS from the cutaneous nerves of the cat, rabbit and guinea-pig [Adrian & Zotterman, 1926; Zotterman, 1939] have given information as to the sensory discharges from the hairs covering the body surface. These discharges are usually of brief duration, occurring only when movement is carried out and ceasing when a steady deflected position is reached. The nerve endings stimulated by hair movement can therefore be described as rapidly adapting, although the short duration of the discharges might be due to mechanical factors, such as the yielding of tissues, rather than to the intrinsic properties of the endings [cf. Dun & Finley, 1938]. But the investigations referred to have been concerned with the covering hairs of the body and limbs; the large vibrissae or tactile hairs of the face have not been specially examined. They might well react differently, since they are supplied by more elaborate nerve endings and have a more definite sensory function than the covering hairs. Indeed, the vibrissae represent a distinct type of organ, absent in man but important in the life of most quadrupeds.

The present work deals primarily with the normal sensory activity of the tactile hairs of the face, studied by the usual technique for recording afferent impulses. In the course of the work it became clear that preparations of these organs gave such consistent results that they could be used for the investigation of problems of more general interest, such as the nature of the adaptation process, its modification by ions, etc. The latter is dealt with in the second part of the paper.

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## HISTOLOGY

A good description of the tactile hairs and their nerve endings has been given by Ramón y Cajal [1933]. The tactile hairs are quite clearly differentiated from the ordinary hairs by the presence of a large cavernous vascular covering in the connective tissue sheath. They have a rich supply of nerve terminations from the many nerve fibres which coil round the hair bulb and run along the side of the hair sheath as a terminal palisade. Under some epithelial cells beneath the vitreous coat, nerve endings are found in the form of circular menisci or ivy-leaf appendages lying perpendicular to the root. Other club-like endings are found parallel to the hair and situated above the vitreous membrane. The hairs of the eye lashes are thought to be the closest example in man of the tactile hairs of the cat, rat, mouse and other animals.

## METHOD

The cat, anaesthetized with dial (Ciba) 0.6 ml./kg., having had a tracheal tube inserted, was laid upon its ventral surface and a screw clamp fixed between the upper jaw and the lower surface of the unwanted eye. The eyelids were removed and the periorbita opened at the reflexion from the eyeball. The eye was quickly removed from the capsule, there being no necessity to ligature the artery accompanying the optic nerve if the eye were removed with sufficient torsion to tear the vessel. The periorbita was then separated from the surrounding fatty tissue and the maxillary, which is the afferent nerve from the vibrissae, exposed lying on the inferior surface of the orbital cavity. The nerve was carefully separated from the accompanying artery and sectioned as close to the brain as possible.

The animal was placed in a heated moist box, the nerve laid upon a thin sheet of ebonite, and dissected with needles. The action currents were recorded with a condenser coupled amplifier and a Matthews oscillograph. The hairs were stimulated either by traction or pressure. The former was provided by a weight attached to a fine silk thread running over a pulley, the thread being tied to the hair by the minimal quantity of sealing wax. The application of the weight by this method was brought about by allowing the weight to drop through a fixed distance without interference. The application of the stimulus could be looked upon as almost immediate. Slower applications could be brought about by allowing the weight to rest upon a lever, the other end of which was attached to a circular disk immersed in a treacle dashpot. For pressure stimulation a cardiac lever was used, a weight being laid on one arm of the lever, the hair being fixed to the other arm. The stimulus here was always applied immediately. It was usually found that the most constant results were to be obtained using the traction method of stimulation but, as will be seen later, this could not be used in every case.

When it was intended to inject solutions into the whisker-bearing area the common carotid artery was selected. The thyroid, internal carotid, occipital and lingual arteries having been tied, a cannula was inserted in a cranial direction. The temperature of injected solutions (all isotonic) was kept at 37°.

## PART I

*Results*

*Nature of effective stimulus. Directional effects.* When the nerve is subdivided it is found that the tactile hairs supplying each bundle of nerve fibres are distributed in circles around the lower surface of the orbit. When the superior hairs, lying close to the orbit, are examined it is usually found that a much larger afferent discharge is produced by bending the hairs down, towards the mouth, than upward to the eye. For the hairs near the mouth the discharge is greater for bending upward than downwards. For the hairs near the snout, bending aborally is more

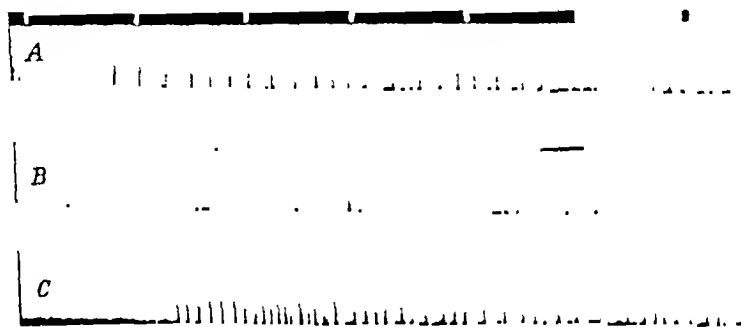


Fig. 1. Different planes of movement stimulating the same nerve ending. *A*=up, *B*=back, *C*=push in. No other movement discharged the ending. Time  $\frac{1}{10}$  sec. All records read left to right.

effective than bending orally, and the reverse is true of the hairs lying farther back. In some nerve fibres a discharge can be obtained by bending the hair in one direction only, but it is more usual to find that movement in at least two directions is an effective stimulus, e.g. in either a forward or an upward direction (Fig. 1). Pulling the hair outwards from the skin is never as effective a stimulus as bending it sideways in the optimal direction, and it was unusual to find that the same ending would be stimulated by pushing the hair in towards the skin and by pulling it outwards. It happened on two occasions that the ending which was stimulated by pulling a hair was stimulated by pulling on more than one hair; in one case pulling on four different hairs seemed to stimulate the same endings (Fig. 2).

This directional sensitivity of the tactile hairs has, no doubt, some functional significance, for the results indicate that the hairs are most sensitive to the particular stimulus which they will be the first to meet. Thus an object moving downward relative to the head will bend the superior hairs first, if it moves aborally it will bend the hairs near the snout before any others, and so on.

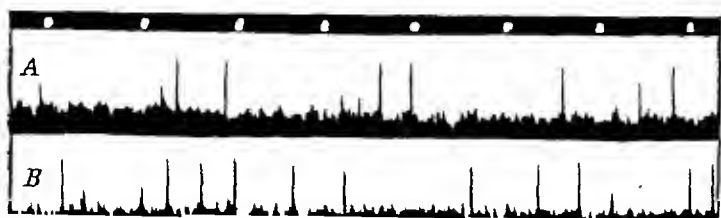


Fig. 2. Both *A* and *B* show response of single ending to stimulating two different hairs. Stimulation of two other hairs also gave responses. Time  $\frac{1}{16}$  sec.

### *Adaptation*

The endings in the ordinary covering hairs of the body respond to movement with a discharge which lasts only a fraction of a second. With the tactile hairs, on the other hand, the endings are in the main slowly adapting. The discharges in single nerve fibres from the region of the vibrissae are sometimes very brief, but in most cases it has seemed probable that the endings giving such discharges were attached to the fine hairs surrounding the larger vibrissae. It is true, however, that on occasions the ending was stimulated maximally through the vibrissae so that we must admit the possibility that some of the tactile hair endings are rapidly adapting.

Typical discharge curves from slowly adapting endings are shown in Fig. 3, the endings respond to the stimulus by a moderately high initial frequency, the value reached depending upon the intensity of the stimulus, i.e. the size of the weights used, and the speed of application (Figs. 3, 4).

In constructing the adaptation curves 15 min. rest was always allowed between tests so as to give the endings time for complete recovery. The endings are not as slowly adapting as those found in muscle by Matthews [1931, 1933] but the curves resemble those for the vagal endings in the lung [Adrian, 1933], there being a gradual decline in frequency with complete failure in about 10 min.

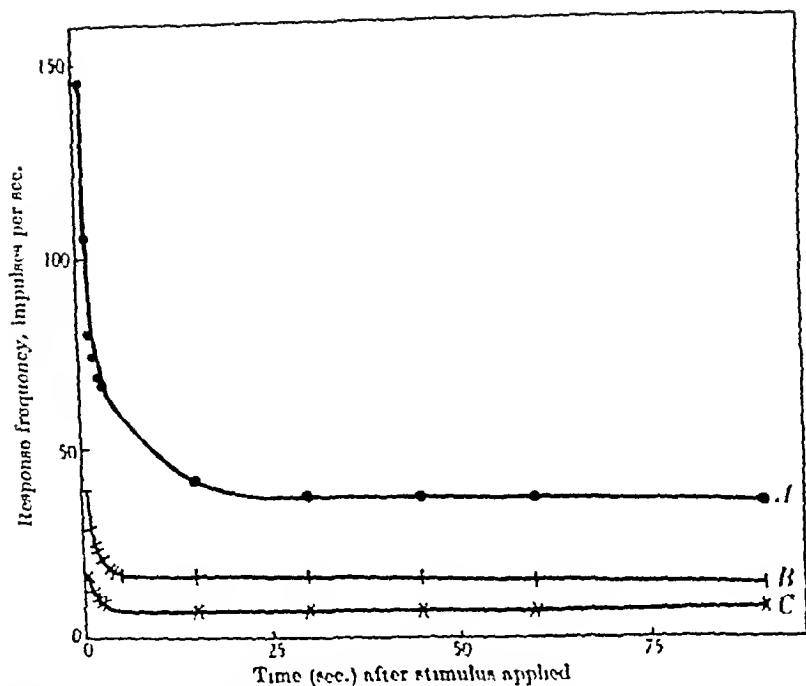


Fig. 3. Effect on discharge curve of varying load. Stimulus applied by traction.  $I = 2$  g.,  $B = 1$  g.,  $C = 0.5$  g.

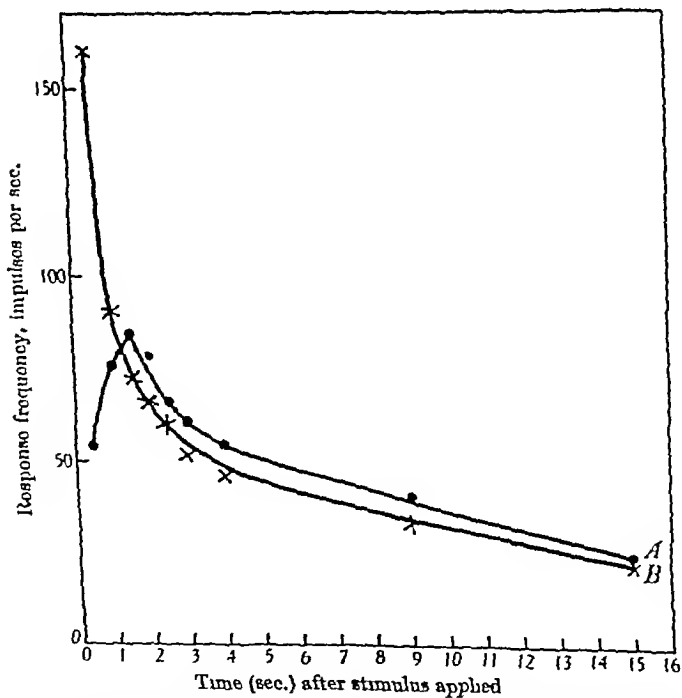


Fig. 4. Effect of different speeds of loading on the discharge curve. Stimulus (2 g.) applied by traction. A = stimulus applied in 1.2 sec. B = stimulus applied in 0.2 sec.

Like the directional sensitivity the slow adaptation of the tactile hairs is probably of value in some of the postural adjustments of the head in relation to solid objects. Although the vibrissae would not be held very long in the bent position, unless the animal were lying down, they might remain distorted for several seconds and it is probably important that there should be very little falling off in the strength of their signals during such a period. Also, by reason of the slow adaptation, a relatively small number of vibrissae can give accurate information of the position of objects in contact with them. Their effectiveness in this respect is shown by Bard's [1938] finding that a blindfolded (but otherwise normal) cat held in the air by the body, reacts to pressure (unilateral or bilateral) of the vibrissae against a solid object by the accurate placing of the forefeet on the object.

#### *Spontaneous discharge*

During the course of cutting down a nerve it was nearly always found that out of a bundle of twenty fibres one or two were in a state of spontaneous activity, and one of these could sometimes be isolated. The number of endings giving a spontaneous discharge is small, usually less than 5% of the total number examined, and the rate of discharge rarely exceeds 40 per sec. It is found that low frequency spontaneous discharges are often more regular than discharges of the same frequency induced by stimulation.

The origin of these spontaneous discharges is of considerable interest, for they have been found with many types of sense organ and their nature has given rise to some speculation. In the present instance the most probable explanation of the discharge is that it is due to a combination of an unusual instability in the ending with an external stimulus which is just above the threshold. The unusual instability is shown by the great regularity at low frequencies (referred to above), and the absence of the usual adaptation, but the second factor, i.e. the intervention of an external stimulus, is strongly suggested by the effects produced by slight movements of the hairs. Thus it is often found that if the hair be moved to the appropriate position all spontaneous activity ceases and does not recur whilst this position of the hair is held. On one occasion it was found that a spontaneous discharge stopped and another ending was stimulated (Fig. 5); when the hair is released the spontaneous discharge recommences with an initial peak in the frequency curve similar to that found normally on stimulation, though of a slighter extent.

Granit [1935] showed the existence of an inhibitory component in a sense organ (the eye). Sand [1937] has described phenomena similar to

those in the present paper of the abolition of a spontaneous discharge and also suggested that they represent an inhibitory process at the sensory nerve ending, comparable to that occurring within the central nervous system. In the present case, at any rate, it seems unnecessary to postulate anything more than the presence or absence of an excitatory stimulus.



Fig. 5. Spontaneous discharge affected by movement. Upper = spontaneous discharge stopped by hair movement and another ending seen to be discharging. The large excursion is an artefact. Lower = on release of the hair the spontaneous discharge recommences with a slight peak. The other ending stops discharging. Time  $\frac{1}{10}$  sec.

The ending is in an abnormally irritable state, a very slight deformation causes a persistent discharge, and the discharge ceases when the stimulus is withdrawn.

#### *The "silent period"*

A well-known accompaniment of the spontaneous discharge is the silent period, the temporary failure of the discharge following increased activity. This was described by Adrian & Zotterman [1926] for the stretch receptors of frog muscle, but the factors upon which it depends were not

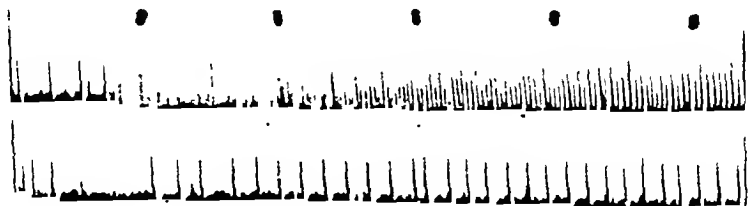


Fig. 6. Spontaneous discharges and the silent period. Upper = stimulation of an ending already spontaneously discharging. Lower = after 1.5 sec. Stimulus is withdrawn resulting in a silent period which is followed by the rebuilding of the spontaneous discharge. Time  $\frac{1}{10}$  sec.

investigated. The effect is shown in Fig. 6, which illustrates both the silent period after the stimulus is withdrawn and the gradual rebuilding of the discharge to the original frequency.



In these experiments the length of the "silent period" has been found to depend upon the total activity produced during the period of stimulation. It does not depend upon the particular frequency occurring at the moment when the stimulus is withdrawn, for it may be seen from Fig. 7

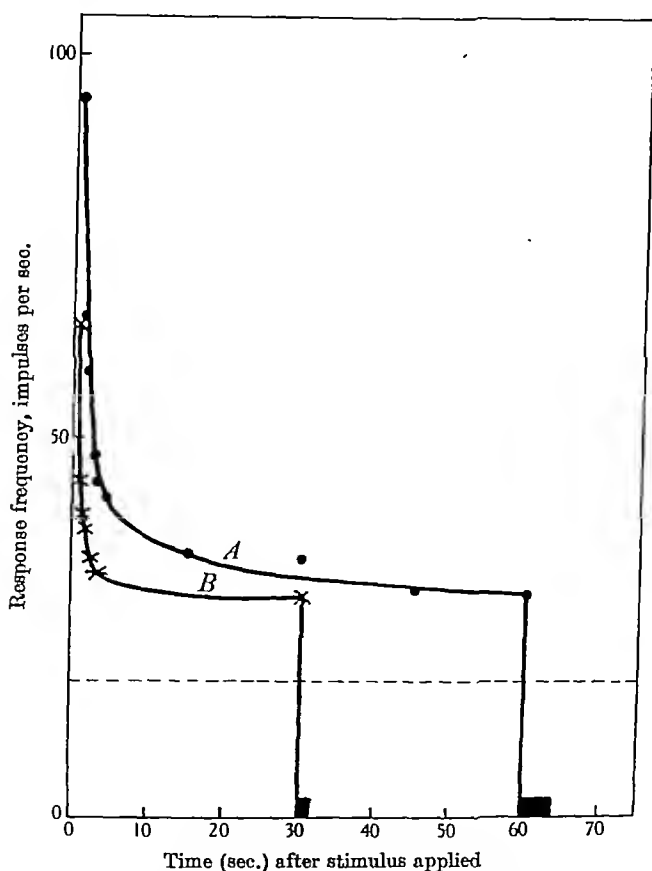


Fig. 7. The greater discharge of the ending leads to greater silent period. Stimulus applied by traction.  $A = 17$  g.,  $B = 7$  g. Blocked out area on base = length of silent period.  $A = 1.0$  sec.,  $B = 3.9$  sec. The intermittent line gives the level of the previous spontaneous discharge.

that similar frequencies at the moment of removal of the stimulus do not lead to similar lengths of silent period. Where stimuli of different intensities were used it was seen that the initial frequency on application of the stimulus was not the sole determining factor, and that the duration of the increased discharge was also important. Finally, in Fig. 7 it will be

seen also that there is a reasonably close relation between the length of the silent period and the total number of impulses set up during the period of stimulation.

After the silent period the spontaneous activity slowly returns along a smooth curve and finally the original frequency of the discharge is again reached. The greater the length of the silent period the more gradual is the curve of recovery, as might well be expected if the failure of the discharge is due to a fall of excitability of the ending, consequent on the period of increased activity.

This gradual return of spontaneous discharge after the silent period is in sharp contrast with the sudden return which takes place when the hair is released from the "no discharge" position (cf. Fig. 5), for then there is an initial peak followed by a decline to a lower level of frequency. In the latter case, presumably, the absence of the discharge was due to the withdrawal of the stimulus which normally maintains it, whereas in the former it was due to a fall of excitability which rendered the stimulus temporarily ineffective; this fall of excitability passes off gradually and allows the stimulus to exert its former effect on the unstable ending.

## PART II

In preparations from the frog the influence of changes in the ionic constituents of the surrounding fluid on the activity of the sensory ending is much the same as on skeletal or cardiac muscle. Thus Matthews [1931] has shown that reduction of  $\text{Ca}^{++}$  leads to a spontaneous discharge of the stretch receptors in frog muscle, and Talaat [1933] has found the same result with endings in frog skin, whilst recently Dun & Finley [1938] have produced very rapid adaptation in the tactile endings of the frog by increasing the  $\text{Ca}^{++}$ . An increase of  $\text{K}^{+}$  depresses and may abolish the response of these endings. Feng [1933] showed that the  $\text{K}^{+}$  liberated from injured skin was enough to make the endings inexcitable, and it has been suggested by Hoagland [1936*a*, Summary] that sensory adaptation in general is brought about by the same mechanism, i.e. that it is due to a fall of excitability caused by the liberation of  $\text{K}^{+}$  from cells near the endings.

With the tactile hair preparation it has been possible to extend this study to mammalian endings, for solutions injected headwards into the common carotid artery will bathe the whisker-bearing area, and it is not difficult to rule out the effects of muscular twitching, etc. The results have been of considerable interest; they show that small changes in certain

ions may have a pronounced effect on adaptation rate, but in the case of  $K^+$  there is an initial stimulation which is hard to reconcile with Hoagland's theory.

*Action of sodium chloride upon the endings*

Small quantities of isotonic sodium chloride appeared to have little or no effect upon the endings, but when approximately 15 ml./kg. of isotonic sodium chloride had been injected into the carotid artery there was

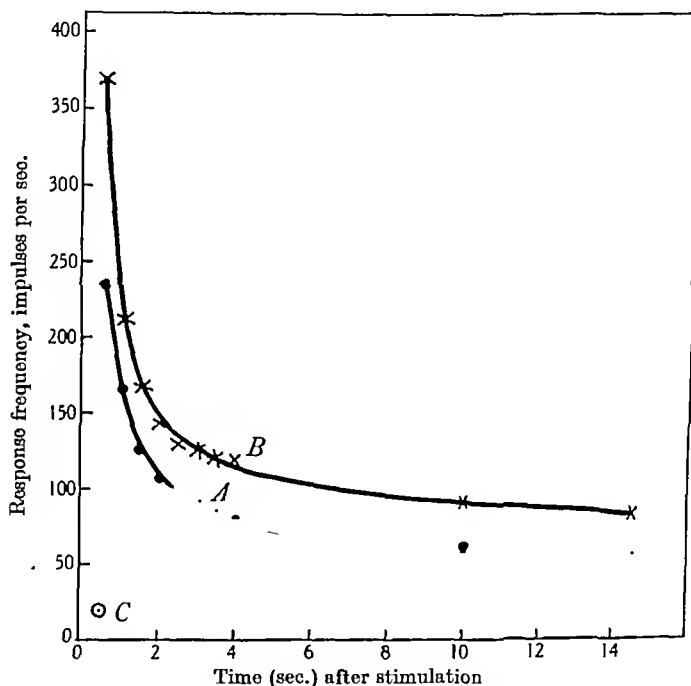


Fig. 8. The increased response of an ending after  $Na^+$  injection with the effect of  $CaCl_2$  upon it. The same strength of stimulus was used in both cases. *A* = before injection. *B* = 4 min. after 135 mg./kg. of  $NaCl$ . *C* = 3 min. after 40 mg./kg. of  $CaCl_2$  given to same preparation.

usually a development of spontaneous activity. This could be seen when there was no twitching round the facial area. It lasted some minutes or even longer and then the effect gradually disappeared. This stimulating action of the sodium upon spontaneous activity was never as plain as that of sodium citrate or  $K^+$  (probably due to the fact that such large or probably variable doses were needed), and was more easily seen when more than one active fibre was present in the nerve bundle. It appeared as if some endings were more easily set into a state of spontaneous

activity than were others. The animal never showed any ill-effects as a result of the injections.

Isotonic sodium chloride in quantities which are sufficient to produce spontaneous discharge from the nerve endings also affect the frequency curve. There is an increased frequency of discharge on stimulation, and the frequency remains at a higher level than normal through the adaptation period (Fig. 8). This effect is quite similar to that found by Matthews [1931] for the muscle spindle of the frog. The stimulating action of  $\text{Na}^+$  upon the ending can be rapidly and completely antagonized by  $\text{Ca}^{++}$  in small doses (Fig. 8). It seems probable that the action of  $\text{Na}^+$  is really due to the relative fall in the concentration of the other ions, for large quantities of  $\text{Na}^+$  must be injected for an effect; much smaller amounts of  $\text{K}^-$  or  $\text{Ca}^{++}$  having marked effects upon the nerve endings.

#### *Action of potassium chloride upon the endings*

As a result of the injection of 1 ml./kg. KCl there is an immediate marked twitching of the facial musculature accompanied by a high-frequency discharge from the nerve endings. This is probably due in part to the hair movement, but when all muscular activity has ceased the ending still continues to discharge spontaneously for at least some minutes. The rate is usually high at first, falling gradually to zero. This is probably due to the washing out of the  $\text{K}^-$  by the blood stream. It is essential to inject the KCl very slowly even in quite small quantities (1-2 ml./kg.), for a sudden sharp injection may lead to cardiac failure. After a slow injection there may be some slight signs of cardiac and respiratory effects but these rapidly disappear.

The effect of  $\text{K}^-$  injection upon the adaptation rate is interesting, for even 10 mg. kg. KCl injected intra-arterially cause a marked increase in the initial frequency, and the discharge through the period following stimulation is much higher than before the injection (Fig. 9). Spontaneous activity as a result of the injection may have ceased, but this effect persists. The effect is somewhat similar to that which Matthews [1931] found for  $\text{Na}^+$  but is the opposite to what he found for  $\text{K}^+$ . A subsequent injection of more  $\text{K}^+$  usually gives a further slight increase in frequency after stimulation, but the maximum increase in initial frequency to a stimulus after the injection has been about 60%.

As can be seen, the level of the discharge remains at a higher frequency all through the period of stimulation. The stimulating action of  $\text{K}^-$  upon the endings seems to be followed by depression, but for the development of this depressive action, large quantities (up to 70 mg./kg. of KCl) had to

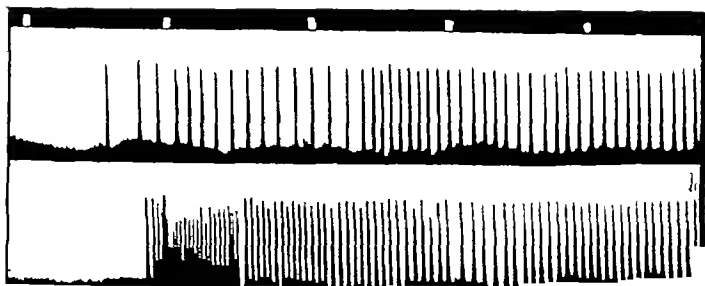


Fig. 9 The modification of the frequency of response by  $K^+$ . Response to 2 g. traction. Upper = before. Lower = 5 min. after 10 mg./kg. of KCl through the artery. Time  $\frac{1}{10}$  sec

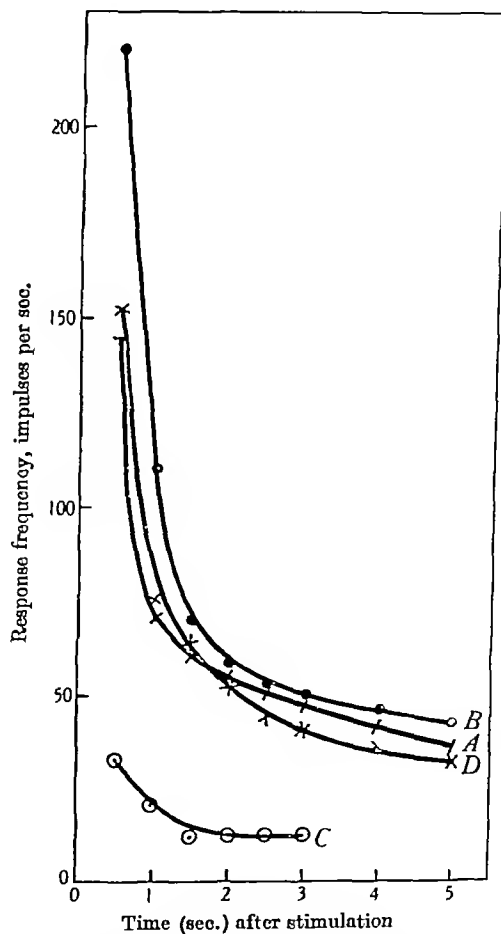


Fig. 10. The stimulating and depressing effects of  $K^+$  with the effect of  $Na^+$  upon the latter. A = before. B = 2 min. after 10 mg./kg. of KCl. C = 5 min. after total of 50 mg./kg. of KCl. D = 25 min. after 150 mg./kg. of NaCl.

be injected and there was always a prolonged initial increase in the frequency on stimulation before the onset of depression. The chief manifestation of depression was a decreased initial response on stimulation with a markedly increased adaptation rate. If the intra-arterial injection had been given at a sufficiently slow rate this depression was not accompanied by other signs of disturbance in the animal.

The antagonist action of various ions to  $K^+$  is of interest.  $Na^+$  did not appear to have any antagonism to the stimulatory effects of  $K^+$ .  $Ca^{++}$  in equal doses prevented the occurrence of the spontaneous rhythm and the increased initial response on stimulation. When the depression after excess  $K^+$  had appeared,  $Ca^{++}$  in quite small amounts led to an even more marked depression of activity. If more  $Na^+$  were injected, in doses of approximately 15 ml./kg. of isotonic  $NaCl$ , there was a gradual return to normal, the ending eventually completely recovering its response to stimulation (Fig. 10). The predominantly depressive action of  $K^+$  found by other workers is possibly due to the fact that they were using frog's tissues and applying the solutions by irrigations and not, as in these experiments, by arterial injection. It must be emphasized that the stimulating effect is not due to the muscular contractions acting on the hairs, for neither slight contraction nor hair movement is observable when the ending is discharging spontaneously. Also, even where there is no further spontaneous activity the frequency response of the ending is much higher than before  $K^+$  injection, showing that the effect is on the ending rather than on the surrounding tissues.

#### *Effect of $Ca^{++}$*

If an ending or a group of endings be discharging spontaneously the injection of a small quantity of  $CaCl_2$  (15 mg./kg.) will stop the discharge. This effect can be seen in Fig. 11. Less  $CaCl_2$  is required when the injection is a close one through the artery, rather than when the solution is injected through the femoral vein, but larger quantities of  $CaCl_2$  (75 mg./kg.) appeared to have no marked toxic action upon the normal animal.

If a quantity of  $CaCl_2$  be injected which is sufficient to stop all spontaneous activity it will usually be found that it has some effect upon the adaptation rate, although a more marked effect can be found with larger doses than that required just to stop spontaneous activity. The effect of 40 mg./kg. of  $CaCl_2$  is shown in the accompanying Fig. 12. In this case it may be noticed that the  $CaCl_2$  solution has been injected through the femoral vein and so the  $Ca^{++}$  was distributed through the body fluid before eventually reaching the ending in the hair. Obviously

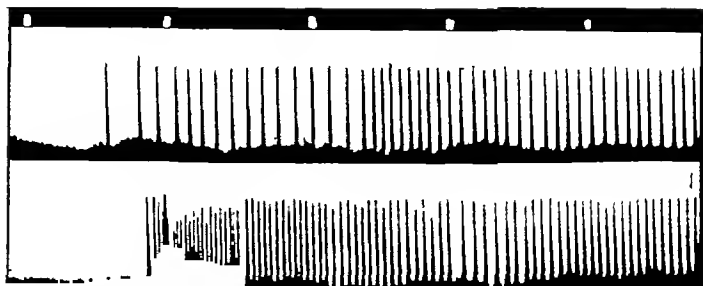


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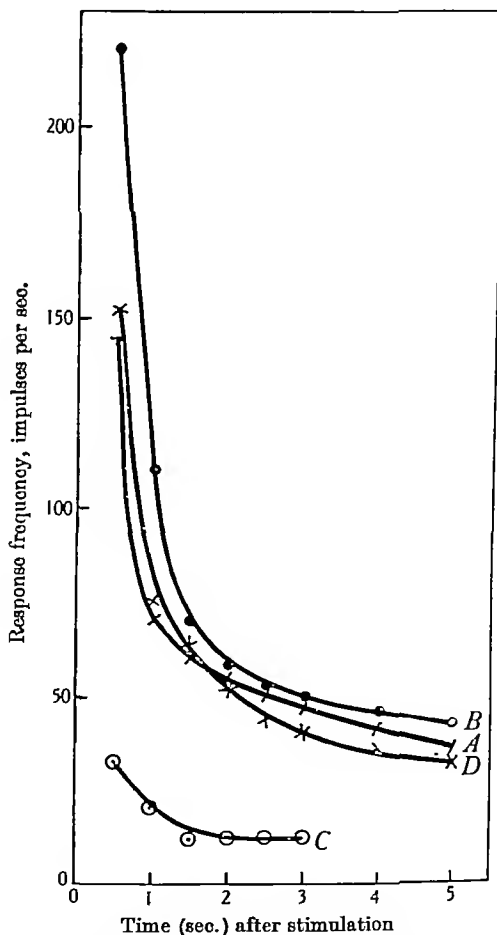


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the effective quantity here was quite small but the effect was a prolonged one. It can be seen clearly in Fig. 12 that there are two distinct effects. The first is not so constantly found and consists in a depression of the initial response. A more constant and possibly more characteristic result is that seen on the adaptation rate, for here the ending is seen to adapt a great deal more quickly than before injection.

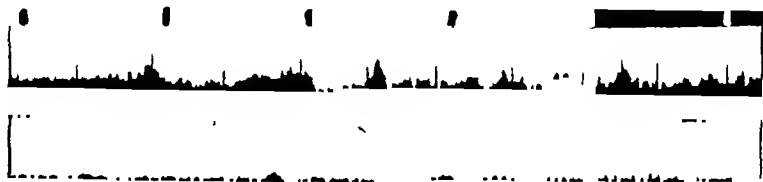


Fig. 11. The effect of  $\text{Ca}^{++}$  upon a spontaneously discharging ending. Upper=before. Lower=1 min. after 15 mg./kg. of  $\text{CaCl}_2$ . Time  $\frac{1}{10}$  sec.

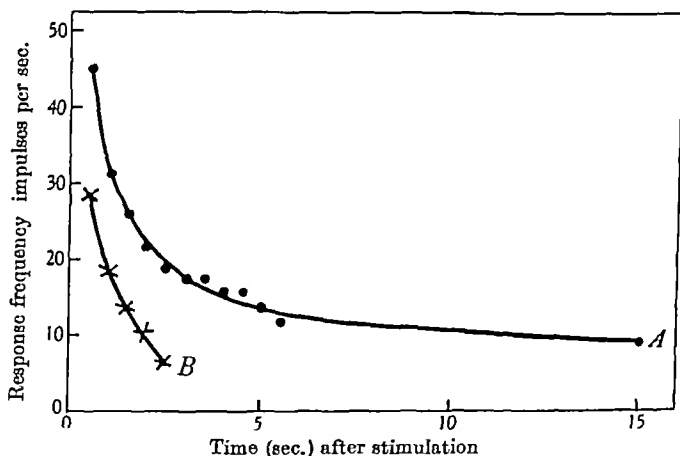


Fig. 12. The depressant action of  $\text{Ca}^{++}$  upon the nerve ending. A=before. B=5 min. after 30 mg./kg. of  $\text{CaCl}_2$  through the femoral vein.

The relation of  $\text{Ca}^{++}$  to other ions is of interest.  $\text{Na}^+$  in large quantities was capable of reversing the depressant effects of  $\text{Ca}^{++}$  upon the ending, but not to the extent, as far as was observed, of development of any marked spontaneous activity, the main effect being a return of the adaptation rate to normal. The initial effect of any quantity of  $\text{K}^+$  is to prevent the depressant action of  $\text{Ca}^{++}$ . Sodium citrate (3% isotonic) led to the development of marked spontaneous activity, as it does in frog's skin. The effect of citrate upon the adaptation time was not investigated.

## DISCUSSION

In these experiments the ion which is held by some workers to be the cause of rapid adaptation at sensory endings appears to stimulate these in no uncertain fashion. 1 ml. kg. of isotonic KCl, injected into the artery supplying the efferent area, caused a prolonged stimulation of the ending. This quantity of KCl, when distributed in the total body fluids of the animal, would seem to be so small that there is presumably some local concentration effect at the ending on injection, with a gradual disappearance of this effect. This view is supported by the fact that the development of the spontaneous rhythm is almost immediate, and gradually falls off in frequency, with the increased initial response remaining as a more prolonged effect.

In these endings at any rate the speed of adaptation is not affected by quantities of  $K^+$ , which cause spontaneous discharge from the ending at the beginning and a more prolonged increased initial response on stimulation. It cannot, therefore, be seriously advanced that a  $K^+$  increase in the external fluid can be a cause of the eventual adaptation of these endings. In the frog's skin the quantities of  $K^+$  used by Hogland [1936b] were large (15 times the normal amount, and such quantities might be expected to bring about a rapid depression, the stimulation being quickly overcome (if it were ever present). This view is supported by the fact that Hogland found that, if the adaptation of an ending (in Hogland's sense) had been hastened by  $Ca^{++}$ , washing with  $K^+$  solution caused a preliminary marked slowing in this adaptation, followed by quickening. Hogland considered that the depressive action of  $K^+$  was its characteristic effect on the sensory endings of the frog's skin. With these mammalian endings, however, the stimulating action of  $K^+$  seems to be its more pronounced effect, the depressive effect occurring at a later stage. The quantities of  $K^+$  required to produce rapid adaptation would be relatively large and it is doubtful if they could be concentrated at the ending sufficiently quickly.

## SUMMARY

1. A method is described for recording sensory nerve impulses from the vibrissae of the cat.
2. The nerve endings are stimulated most effectively by movement of the vibrissae in particular directions, thus downward movement is most effective for the upper vibrissae and upward movement for the lower.
3. The endings are slowly adapting, the frequency of the discharge varying with the extent and rate of development of the main movement.

4. Spontaneous discharges from the endings occur and they appear to be due to an abnormal irritability of the ending, associated with a stimulus which is just above the threshold.

5. In a nerve fibre giving a spontaneous discharge the length of the silent period following stimulation appears to be related to the total number of impulses discharged during stimulation, rather than to either the initial or final discharge frequency.

6. After the silent period the spontaneous discharge returns gradually to its former value. The shorter of two silent periods is followed by a quicker return to the previous level of spontaneous discharge than is the longer.

7.  $\text{Na}^+$  in large amounts leads to spontaneous discharges and an increased initial discharge.

8.  $\text{K}^+$  in much smaller amounts has a similar effect. In large amounts it has a depressant action upon the spontaneous and the initial discharge, and it also leads to a rapid adaptation of the ending. Citrate produces spontaneous discharge like  $\text{K}^+$ .

9.  $\text{Ca}^{++}$  inhibited spontaneous discharges from an ending, decreased the initial response on stimulation, and quickened the rate of adaptation.

10. The result with the above ions does not agree with the view that  $\text{K}^+$  can be an agent which would produce rapid adaptation in these endings.

I wish to express my sincere thanks to Prof. Adrian, who suggested this work, for his constant advice and criticism. I also wish to thank Dr B. H. C. Matthews for much technical help and advice. The Medical Research Council of Ireland is to be thanked for a grant towards expenses.

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## TRANSMISSION OF LIGHT BY THE EYE MEDIA

By K. J. W. CRAIK

*From the Psychological Laboratory, Cambridge**(Received 19 December 1939)*

STILES & CRAWFORD [1933] discovered that subjective brightness is not proportional to pupil area in man. If the pupil be naturally or artificially constricted to half its area, the physical illumination of the surface viewed need not be increased twofold but by some smaller amount, in order that it shall seem as bright as before. They measured the actual effectiveness of the different parts of the pupil in contributing to the total subjective brightness by traversing a small pencil of light across the pupil, and found that light entering the eye near the margin of the pupil is only about one-quarter as effective as that entering through the central part. They concluded that this effect was probably not due to greater light absorption by the marginal part of the lens etc., but to variations in the directional sensitivity of the retina. Since a pencil of light entering at the margin of the pupil from a centrally fixated object falls obliquely on the retina, they suggested that the structure of the receptors, or perhaps of the retinal pigment, is such as to reduce the sensitivity to oblique rays. That the phenomenon originates in the retina and not in the eye media is rendered probable by the fact that the effect is absent with low intensity light of 430-5  $m\mu$ , falling on the dark adapted parafovea [Stiles, 1939].

It seemed desirable to test this hypothesis by direct measurements of the transmission by the central and marginal parts of the lens and cornea in isolated eyes.

## APPARATUS AND METHOD

The eyes of twelve cats were used. They were extracted immediately after the animals had been anaesthetized. A small window was cut in the back of the eye. In order to avoid loss of vitreous a splinter of microscopic cover glass was first inserted through the sclera and choroid so as to pass into the eye at one place and out at another; this provided a glass shield which remained when the window was cut. The eye (*a*, Fig. 1) was

then mounted with plasticene in a metal frame *b*, capable of universal movement, and the image of a 500 W. projector bulb filament *c*, 1 m. distant, was centred in the window by the help of a loupe *d*. A mask *e* with a 0.5 mm. hole was then lowered just in front of the cornea; it could be traversed across the eye in a slide controlled by a micrometer *f*. A selenium oxide generator type photocell *g* was also placed in a slide at the back of the frame and connected to a galvanometer having a sensitivity of 1050 mm./ $\mu$ A. on a scale 1 m. distant. The eye was frequently moistened with warm Ringer solution. The mask was then traversed across the eye

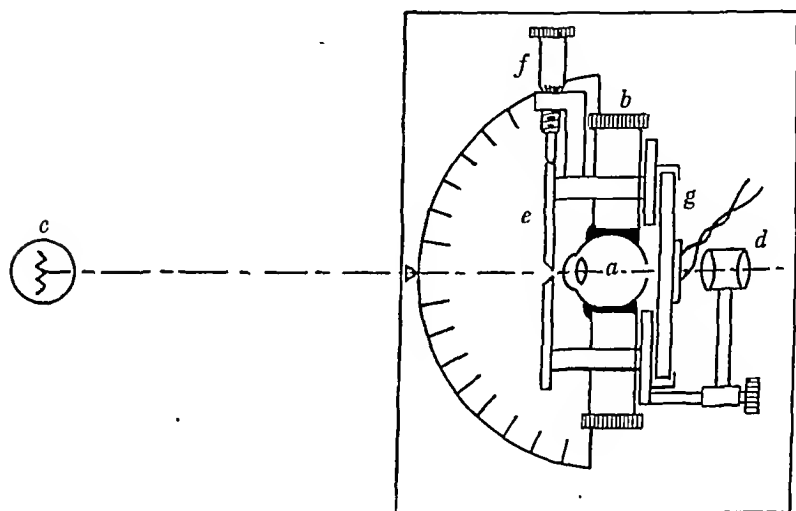


Fig. 1. Apparatus. *a*, eye; *b*, frame; *c*, lamp; *d*, loupe; *e*, mask with hole; *f*, micrometer; *g*, photocell.

so that the hole passed from one pupillary margin to the other; the image of the filament of course maintained a constant position, except for the effects of aberrations of the lens and error in accommodation. Readings were taken approximately every 0.25 mm. across the pupil, followed by a return traverse. The technique of employing a photocell to record the light traversing the media of the eye has been used by Ludvigh & McCarthy [1938].

The cat's pupil dilates widely after removal of the eye and begins to contract again after about an hour. The eyes of two species of frog, *Rana temporaria* and *R. esculenta* were also employed, though their small size made the results less accurate. These pupils contract after excision and were dilated by soaking in 0.01% adrenaline. The moment when the

pencil of light passed from the pupil to the iris could be detected, and the effective pupil diameter measured, since the pencil was clearly visible by scattered light when it fell on the iris.

The mask was then moved to the point on the pupil giving maximum sensitivity and the reduction in deflexion noted when neutral filters having transmission factors of 25, 50 and 75% were placed in front of the hole. This permitted conversion of the galvanometer deflexions directly into relative light intensities, by a graph based on the deflexions at 0, 25, 50, 75 and 100%.

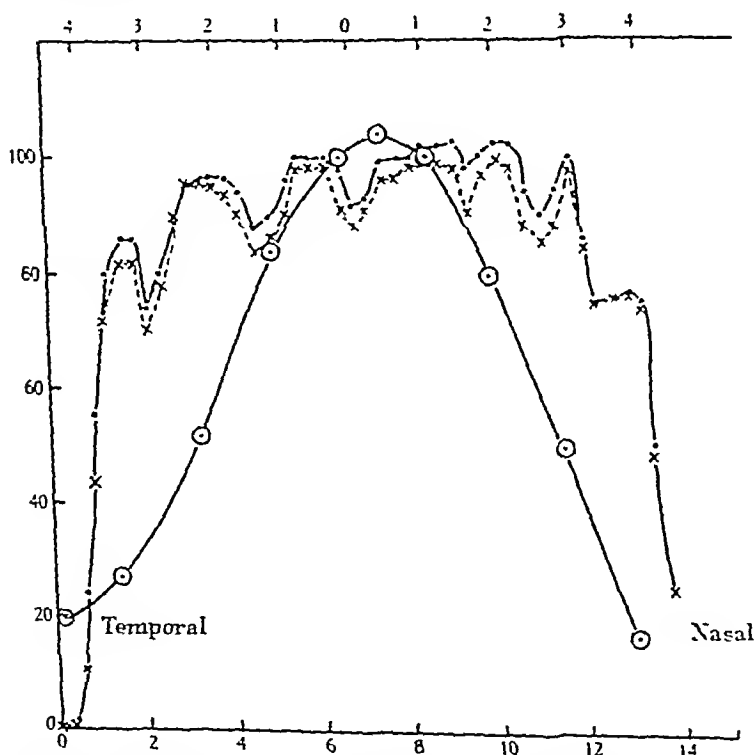


Fig. 2. Horizontal traverse. Dots, first traverse, crosses, second, showing relative transmissions across the pupil. Circles, Stiles & Crawford effect on man. Abscissae: below, mm. across pupil of cat; above, mm. across pupil of man, from Stiles & Crawford's results. Ordinate: percentage transmission of cat media, and percentage luminous efficiency in man.

### RESULTS

Figs. 2 and 3 show the result of a horizontal and vertical traverse across a cat pupil, with Stiles & Crawford's curves superimposed. The scale of the abscissae has been adjusted so that points representing the

pupil diameters on the two sets of curves coincide. This would appear to be the most justifiable way of comparing the two results. It will be seen that the transmission at the margin never falls more than 30% below that at the centre, whereas a drop of 70% or more in sensitivity is found in the genuine Stiles & Crawford effect, measured subjectively in man. Further, the drop in sensitivity at the margin is much more sudden in the present experiments. For instance, over 0.8 of the pupil width, the

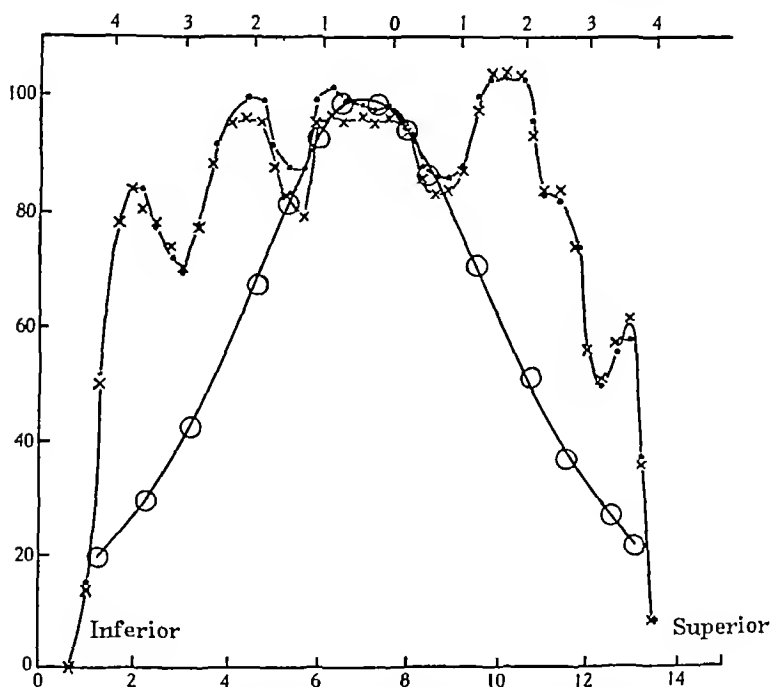


Fig. 3. Vertical traverses. Ordinates and abscissae as in Fig. 2.

sensitivity does not vary by more than 10%, and even this variation is irregular, whereas in the Stiles & Crawford effect the variation over a similar proportion of the maximum human pupil width is approximately 60%, and consists of a gradual decline on either side of the axial point.

Admittedly the present experiments may not relate directly to the human eye. It is unlikely that the cat shows a retinal Stiles & Crawford contract again as it possesses an iridescent *tapetum* in place of a pigment *temporaria* and its vision appears to be an all-rod retina. But the properties made the result comparable. Indeed, as the cat's pupil were dilated very twice as much as the human one—a pupil 12 mm. in

diameter in an eye two-thirds the size—any effect due to the optical media might be expected to be more noticeable than in the human eye. Its small magnitude in spite of this suggests that in the human eye the effect is almost entirely due, as suggested by Stiles & Crawford, to variations in the directional sensitivity of the retina.

The eyes of *R. temporaria* and *R. esculenta* also showed a drop in transmission varying from 12 to 25%. With these smaller eyes it is

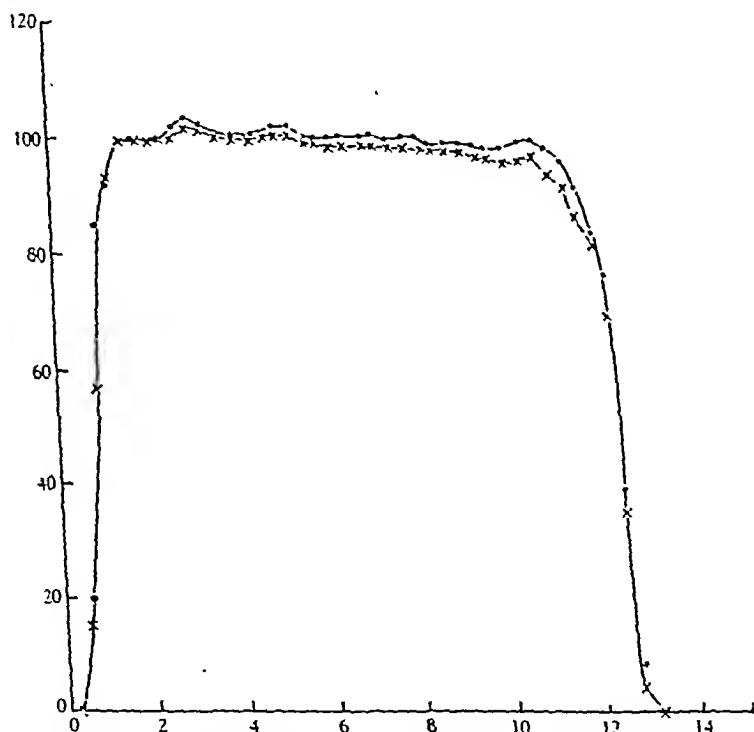


Fig. 4. Traverses on a glass control. Scales: ordinates and abscissae same as in Fig. 2.

difficult to ensure that the pencil of light does not graze the edge of the pupil, following a chord instead of a diameter, in its passage. Also, the pupil is smaller in comparison with the pencil, and the variation in retinal illumination while it enters the pupil consequently occupies a greater proportion of the total traverse.

To indicate the errors of the method of measurement and of factors such as drift due to photocell fatigue, a control curve was taken on an imitation eye, with a glass lens whose focal length and aperture were similar to that of the cat (Fig. 4).



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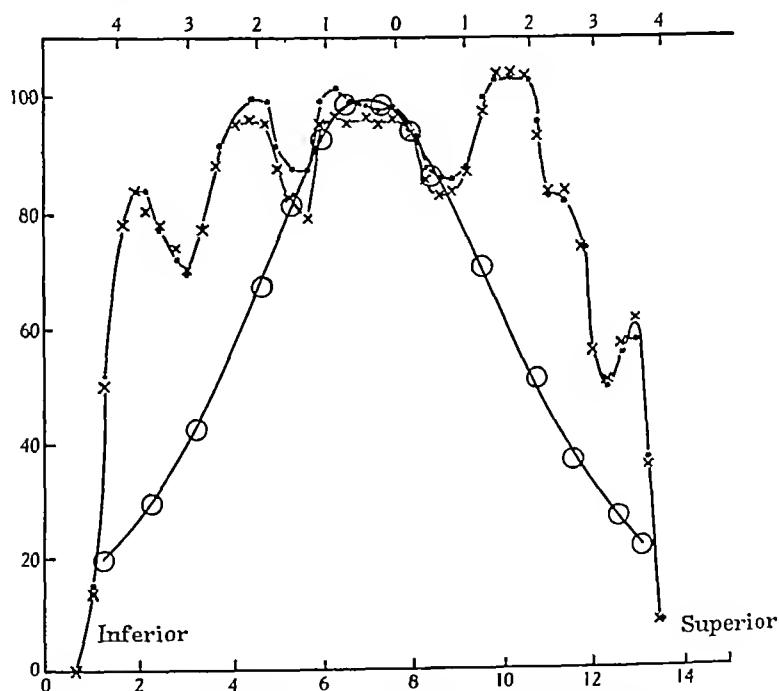


Fig. 3. Vertical traverses. Ordinates and abscissae as in Fig. 2.

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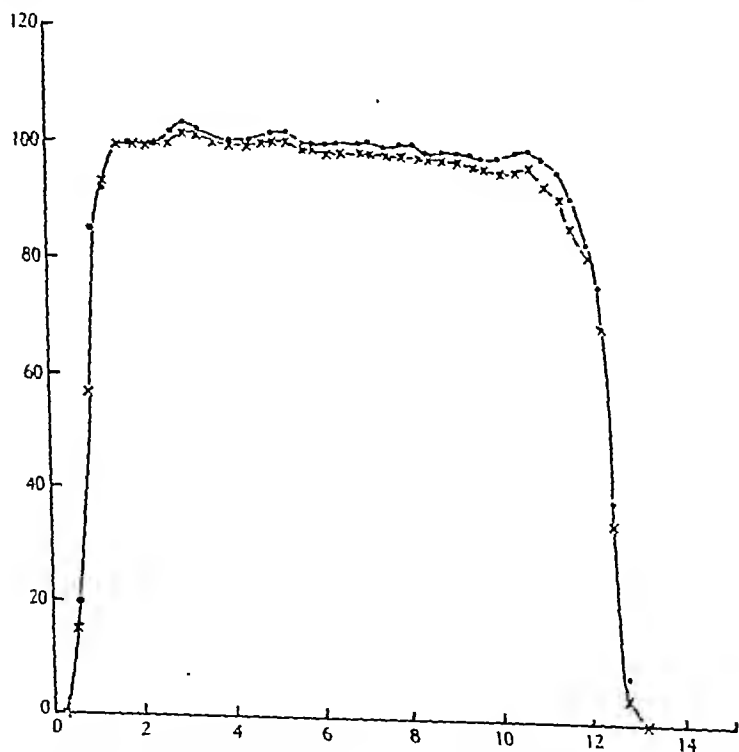


Fig. 4. Traverses on a glass control. Scales: ordinates and abscissae same as in Fig. 2.

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To indicate the errors of the method of measurement and of factors such as drift due to photocell fatigue, a control curve was taken on an imitation eye, with a glass lens whose focal length and aperture were similar to that of the cat (Fig. 4).

It is difficult to know how far the irregularities in the transmission curve are exaggerated by post-mortem changes in the eye, or to inadequate control of temperature and humidity. Visible striations certainly appear in the cornea if the intra-ocular pressure has fallen by loss of vitreous, or if the eye has become dry; but many of these measurements, of which the figures shown are typical, were made within ten minutes of excision, and the irregularities showed great constancy in any one eye, suggesting that they are characteristic of the living animal.

#### SUMMARY

Stiles & Crawford's explanation of their effect, in terms of directional sensitivity of the retina, is corroborated by direct measurements of the relative light transmission at the margin and centre of the lens etc. in the excised eyes of the cat. Persistent irregular variations in transmission up to 10%, and a steady decline at the extreme margin, were found, but the curve is totally different from that obtained by subjective investigation in man, the discrepancy being often as much as 50%.

I am indebted to Messrs Fitzgerald and Pfaffmann of the Cambridge Physiological Laboratory for providing me with freshly excised cat's eyes removed in the course of their own experiments.

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## THE EFFECT OF MONO-iodoacetic ACID ON THE ABSORPTION OF WATER FROM GLUCOSE SOLUTIONS IN THE SUBARACHNOID SPACE

By T. H. B. BEDFORD

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*(Received 8 January 1940)*

THE effect of the monosaccharides, glucose, mannose and xylose, on the absorption of water from the subarachnoid space has been studied in an earlier series of experiments [Bedford, 1939]. The sugars were found to have similar effects on the absorption of water when administered in concentrations of equal osmotic strength. It was suggested as a result of these experiments that the sugars themselves are probably absorbed by a common process. No evidence was discovered of an absorption of glucose by a specific process such as Verzar & McDougall [1936] found to occur in the intestine. In the following experiments an attempt has been made to determine whether mono-iodoacetic acid has any effect on the absorption of water from glucose solutions in the subarachnoid space. According to Verzar & McDougall, mono-iodoacetic acid inhibits the specific forces which accelerate absorption of glucose from the intestine, while processes of simple diffusion, such as those involved in the absorption of mannose and xylose, remain uninfluenced.

### METHODS

Dogs anaesthetized with intratracheal ether were used throughout the experiments. A constant pressure was maintained in the subarachnoid space. In one group of experiments the pressure was 300 mm., and in another 400 mm. of normal saline solution. Higher pressures were not used as they were considered to depart too far from the normal. The methods by which the pressures were maintained and the rate of inflow determined have been described in earlier papers [Bedford, 1938]. The mono-iodoacetic acid in a 10% solution in water was injected subcutaneously. By this means any direct injury to the endothelium of the subarachnoid space was avoided. It was administered in a proportion

of 0.12-0.16 g. per kg. body weight; this gave a calculated concentration for the whole body of 1 : 8000 to 1 : 6000. These concentrations are the same as those used by Verzar & Laszl [1935] in their study of the effect of mono-iodoacetic acid on absorption of sugar from the intestine of rats. The animals were allowed to rest for 1 hr. after injection in a darkened room, when they were anaesthetized and the needle of the recording apparatus introduced into the subarachnoid space. A reading of the rate of inflow was taken at intervals of 3 min. for a period of  $1\frac{1}{2}$  hr. The systolic blood pressure was recorded throughout the experiments and all experiments in which the pressure fell below 100 mg. Hg were discarded. Considerable variation was noted in the reaction of different dogs to the injection of mono-iodoacetic acid in the quantities indicated. In some instances subcutaneous injection was followed after a few minutes by vomiting, purgation and a profound fall in systolic blood pressure. These effects can generally be avoided, however, if care be taken to use only healthy animals and to disturb them as little as possible during and after the administration of the acid. It was generally found impracticable, however, to extend the experiment beyond  $1\frac{1}{2}$  hr. owing to the depressant action of the mono-iodoacetic acid on the circulation. The injection site did not appear to be spontaneously painful up to the time of anaesthetizing although a variable amount of serous exudate was always found on examination after death.

## RESULTS

### *The effect of mono-iodoacetic acid on the absorption of normal saline solution*

A series of six experiments was first performed to determine the effect of mono-iodoacetic acid on the absorption of normal saline solution.

TABLE I. The effect of mono-iodoacetic acid on the rate of absorption of normal saline solution from the subarachnoid space. The readings were begun 1 hr. after the administration of the acid. A pressure of 400 mm. normal saline solution was maintained in the subarachnoid space. Inflow is expressed in c.c. introduced during a period of 3 min. at intervals of 9 min. The readings are correct to the nearest 0.1 c.c. In the actual experiments a reading was taken every 3 min. Exps. 1 and 2 are with control animals which had not previously been treated with mono-iodoacetic acid.

Exp. no.	Mono-iodoacetic acid	Time (min.)											
		0	9	18	27	36	45	54	63	72	81	90	
1	0	0.4	0.4	0.5	0.5	0.4	0.6	0.4	0.5	0.4	0.5	0.4	
2	0	0.5	0.3	0.5	0.4	0.4	0.5	0.4	0.5	0.5	0.6	0.5	
3	1/6000	0.6	0.6	0.7	0.5	0.7	0.6	0.5	0.5	0.5	0.5	0.5	
4	1/6000	0.4	0.3	0.4	0.5	0.4	0.3	0.5	0.5	0.5	0.4	0.5	

In four the subarachnoid pressure was maintained at 400 mm. normal saline solution and in the remainder at 300 mm. The rate of inflow was found to be the same as that observed in animals which had not previously been poisoned with the acid (Table I).

*The effect of mono-iodoacetic acid on the absorption  
of glucose solutions*

*Glucose in isotonic concentration.* Isotonic glucose solution (5.4%) was introduced into the subarachnoid space 1 hr. after poisoning with mono-iodoacetic acid. Six experiments were performed at a constant pressure of 400 mm. and four at a pressure of 300 mm. normal saline solution. No effect was observed on absorption except in two of the experiments performed at 400 mm., when a slight increase was noticed towards the end.

*Glucose in twice isotonic concentration.* Four experiments were performed using glucose solution of twice isotonic concentration. The subarachnoid pressure was maintained at 300 mm. normal saline solution.

TABLE II. The effect of mono-iodoacetic acid on the rate of absorption of isotonic glucose solution from the subarachnoid space. The glucose was introduced at a pressure of 400 mm. normal saline solution 1 hr. after administering the acid. The data have been treated in the same way as in Table I. Exps. 1 and 2 are with control animals which had not previously been treated with mono-iodoacetic acid.

Exp. no.	Mono- iodoacetic acid	Time (min.)										
		0	9	18	27	36	45	54	63	72	81	90
1	0	0.5	0.6	0.4	0.6	0.4	0.5	0.4	0.6	0.4	0.6	0.5
2	0	0.4	0.4	0.6	0.6	0.5	0.4	0.5	0.4	0.6	0.5	0.5
3	1/7000	0.5	0.3	0.4	0.5	0.5	0.4	0.4	0.4	0.5	0.5	0.5
4	1/6000	0.4	0.4	0.5	0.3	0.3	0.4	0.3	0.3	0.4	0.3	0.4
5	1/6000	0.7	0.8	0.8	0.9	0.7	0.8	0.8	0.9	0.8	0.8	0.9
6	1/7000	0.6	0.5	0.4	0.5	0.5	0.5	0.5	0.6	0.5	0.6	0.6
7	1/6000	0.4	0.6	0.4	0.5	0.4	0.4	0.5	0.4	0.4	0.5	0.4
8	1/6000	0.4	0.3	0.2	0.4	0.4	0.5	0.5	0.4	0.5	0.5	0.5

TABLE III. The effect of mono-iodoacetic acid on the rate of absorption of twice isotonic glucose solution from the subarachnoid space. The glucose was administered at a pressure of 300 mm. normal saline solution 1 hr. after administration of the acid. The data have been treated in the same way as in the previous tables. The first two experiments are with control animals which had not previously been treated with the acid.

Exp. no.	Mono- iodoacetic acid	Time (min.)								
		0	9	18	27	36	45	54	63	
1	0	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	
2	0	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	
3	1/6000	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	
4	1/6000	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.1	
5	1/6000	0.4	0.3	0.4	0.4	0.4	0.2	0.1	0.1	
6	1/7000	0.3	0.2	0.2	0.2	0.2	0.2	0.1	0.1	

A reduction in the rate of inflow was observed which resembled very closely in its extent and time of onset that found in animals not previously poisoned with mono-iodoacetic acid. Figures from experiments illustrating the action of mono-iodoacetic acid on absorption of glucose are given in Tables II and III.

### DISCUSSION

Although it would appear that mono-iodoacetic acid is without influence on the absorption of water from glucose solution when the latter is introduced into the subarachnoid space at pressures of 300 and 400 mm. normal saline solution for  $1\frac{1}{2}$  hr., it is questionable to what extent the experiments justify the conclusion that glucose is normally absorbed from the subarachnoid space by a simple physical process. The concentration of glucose used throughout the experiments was never less than 5.4 %; this is many times the concentration normally present in cerebrospinal fluid. The experiments do not exclude the possibility that small quantities of glucose may be absorbed by a vital process. It was necessary also to employ abnormally high subarachnoid pressures in order to obtain a measurable inflow of fluid. Although these pressures were maintained at as low a level as practicable, they would tend to favour a physical rather than a vital factor in absorption. It might be thought that the rate of inflow and the duration of the experiments were insufficient to obtain complete replacement of the contents of the subarachnoid space. It is doubtful, however, if the experiments can be criticized on this basis. Weed [1935], while studying the effect of foreign solutions on the rate of absorption from the subarachnoid space, found that a period of 10–15 min. was needed in order to establish a new rate of absorption. He used a pressure 400 mm. above the normal; this would give a total subarachnoid pressure of 500–600 mm. normal saline solution. A study of his figures for the minute rate of absorption of Locke's solution reveals that the inflow at 600 mm. was exactly double that at 400 mm. Even at 500 mm. inflow was still  $\frac{2}{3}$  greater than at 400 mm. In the experiments under consideration the subarachnoid pressure never exceeded 400 mm. normal saline solution. The average inflow of glucose per min. solution was somewhat greater than that found by Weed for the absorption of Locke's solution at the same pressure; in some instances Weed's figures were greatly exceeded. It would seem justifiable to conclude that a complete replacement was effected within 20–30 min. in experiments conducted at a pressure of 400 mm. normal saline solution. Ample time would be available therefore for the glucose solution to exert

its full effect at the site of absorption, for the total duration of the experiments was  $1\frac{1}{2}$  hr.

To sum up, the experiments would appear to provide evidence that glucose solution can be absorbed from the subarachnoid space by a process of simple diffusion and filtration. The possibility that small quantities of glucose are normally absorbed by a specific process has not, however, been entirely excluded.

### SUMMARY

1. A study has been made of the effect of mono-iodoacetic acid on the absorption of water from isotonic and twice isotonic glucose solutions in the subarachnoid space.

2. The rates of absorption after  $1\frac{1}{2}$  hr. at pressures of 300 and 400 mm. normal saline solution were the same as those observed in animals which had not previously been poisoned with the acid.

3. It is concluded that the experiments provide additional evidence that glucose can be absorbed from the subarachnoid space by a simple physical process.

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# THE PART PLAYED BY THE RENAL NERVES IN THE PRODUCTION OF WATER DIURESIS IN THE HYPOPHYSECTOMIZED AND DECEREBRATE DOG

BY L. E. BAYLISS AND A. BROWN

*From the Department of Physiology, Edinburgh*

*(Received 15 January 1940)*

It is now generally recognized that the diuresis which results from the rapid ingestion of a relatively large quantity of water is produced through the mediation of some structure or structures in the hypothalamic region of the brain. The work of Verney and his associates strongly suggests that one of the important structures is the posterior lobe of the pituitary body. Fee, and others, however, have shown that complete removal of the pituitary body does not necessarily prevent the onset of water diuresis, and, as a result of his experiments on decerebrate and hypophysectomized dogs, he suggested [1929] that the renal nerves might play a part; this suggestion was supported by the experiments of Bayliss & Fee [1930] on the perfused innervated kidney. It is true that Bykow & Alexejew-Berkmann [1931], confirmed by Klisiecki, Pickford, Rothschild & Verney [1933] have shown that in the intact dog, denervation of the kidney has no action on the response to a subsequent dose of water, but it is conceivable that this might apply only to the animal with normal pituitary body, and not to the hypophysectomized and decerebrate preparation.

The first of our series of experiments was performed exactly as described by Fee [1929], with the addition that the nerves supplying the left kidney were removed as completely as possible either just before or just after decerebration. No conclusive results were obtained, and it was felt that the rather extensive abdominal interference, together with the shock of decerebration, left the preparation in an unsatisfactory condition for studying the response to the administration of water.

In the present series, therefore, the denervation was carried out not less than 10 days before the hypophysectomy and decerebration, and the test of the response to the administration of water.

## METHODS

The denervation was carried out under pernocton and ether, with full aseptic precautions. The left kidney was approached through an abdominal incision and entirely freed from all its attachments except the renal artery and vein and ureter; these were then carefully cleared of all visible nerves, and the kidney fixed to the abdominal wall by a stitch through the capsule at the lower pole.

Before decerebration, or immediately after, the ureters were exposed under ether anaesthesia extra-peritoneally, and catheters were passed up them for collection of urine. (It was found that simple cannulation was apt to be unsatisfactory owing to the possibility of kinking when rigidity came on.) In addition, a bleeding cannula was inserted into one carotid artery and a cannula for recording the blood pressure in the other; a loop of intestine was exposed and either a loop of string tied loosely round it, so that a needle could subsequently be easily inserted, or a glass cannula was tied into it with a purse-string suture; finally, a cannula was often inserted into one jugular vein, as it was not infrequently found that, owing to the haemorrhage encountered during decerebration, intravenous infusion of defibrinated blood was advisable.

Hypophysectomy and decerebration were carried out as follows. Both sides of the skull and both cerebral hemispheres were removed as completely as possible; the brain stem was lifted so as to expose the pituitary body which was then removed, usually intact, with fine forceps; the brain stem was then severed by a cut passing from about the superior corpora quadrigemina to the pituitary fossa; the whole of the brain stem anterior to this was removed and the skull lightly packed with cotton wool. Haemorrhage was controlled by irrigation with hot saline solution, and the field of operation was kept clear by constant suction.

The rate of urine flow was followed continuously. Samples of blood and urine were taken at intervals and analysed for creatinine and chlorides, creatinine hydrochloride being injected subcutaneously so as to raise the blood concentration to a suitable value (10–20 mg./100 c.c.). In addition, in some experiments the total solid concentration of the blood and the total phosphate concentration of the blood and urine, were estimated. (We are indebted to Mr S. R. Elsdon for these last determinations.) Creatinine was estimated as creatinine picrate, chlorides by a slight modification of the electrometric titration method of Eggleton, Eggleton & Hamilton [1937] and phosphates by the method of Fiske & Subbarow [1925].

Water was administered direct into the small intestine from a Mariotto bottle at the rate of about 10 c.c./min.; too rapid administration was apt to lead to regurgitation into the stomach and considerable delay in absorption.

## WATER DIURESIS IN THE DENERVATED KIDNEY

The response to the administration of water was examined in eighteen dogs with one kidney denervated, and in ten control dogs with both kidneys innervated. Water diuresis was obtained as frequently in the denervated kidneys as in the innervated, and altogether no consistent difference was observed between the two groups (Fig. 1). It appears that even in the hypophysectomized and decerebrate preparation, the renal nerves play no part in the production of water diuresis.

The results are expressed concisely in Table I, in which are included, for comparison, the results obtained in the preparations with acutely

denervated kidneys, and some results obtained earlier by Fee and by Bayliss and Fee. It will be seen that an unmistakable response to the

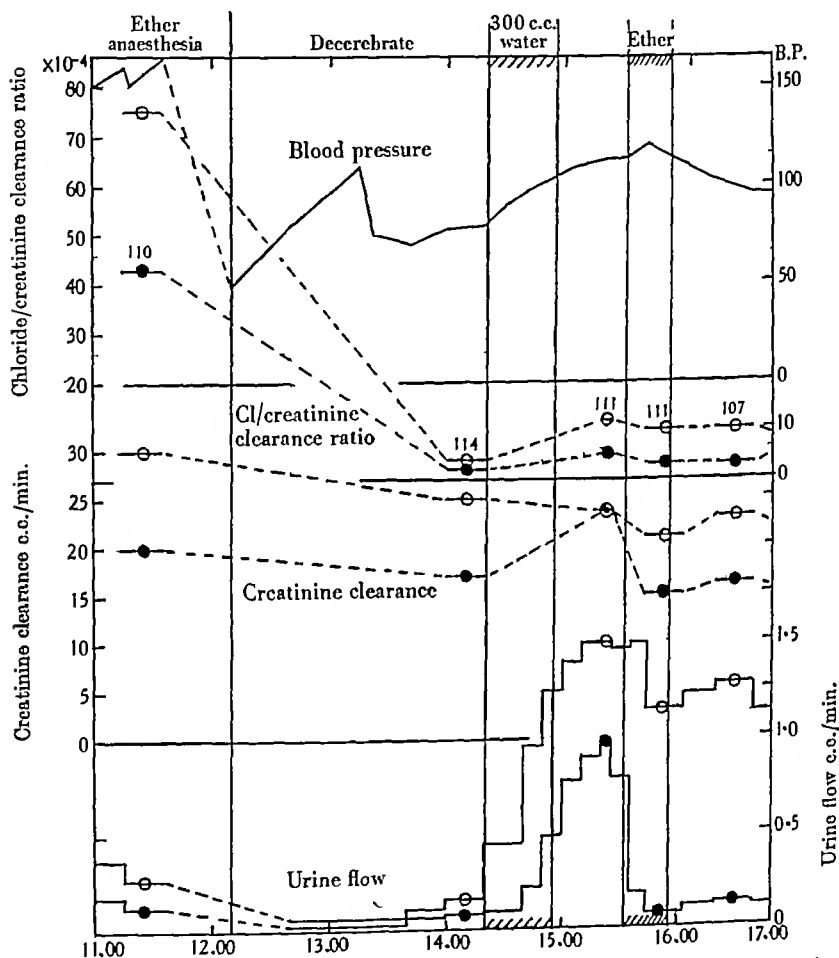


Fig. 1. Dog 27, 10 kg. Left kidney denervated 16 December 1938. Hypophysectomized and decerebrated 9 January 1939. *Water diuresis*, with fall in creatinine clearance in denervated kidney; checked by ether, immediately in innervated, and less markedly, after delay, in denervated kidney; fall in creatinine clearance greater in innervated than denervated kidney. Marked fall in chloride excretion on decerebration. In this and subsequent figures, the numerals against the plots of the chloride/creatinine clearance ratio indicate the plasma chloride concentration (mM.) at that time. ○ denervated kidney; ● innervated kidney.

administration of water was not by any means always obtained (as was observed on similar preparations by Newton & Smirk, 1934). Chronic

TABLE I

Number of experiments showing									
	Water diuresis					No water diuresis			
	Certain		Doubtful		Low blood pressure	Other causes		Total number of experiments	
	Imm.	Dom.	Imm.	Dom.		Imm.	Dom.		
Left kidney denervated:									
Arterio-venous	0	0	1	2	1	4	4	3	9
Cerebro-venous	4	5	7	7	7	7	6	6	24
Total	4	5	8	9	11	11	10	9	33
Both kidneys innervated:									
Lumbal			5		3		11		28
Cerebro			2		5		4		13
Total	11		7		8		15		41
Percentage of total experiments showing water diuresis									
	Certain		Certain-doubtful						
	Imm.	Dom.	Imm.	Dom.	Imm.	Dom.	Imm.	Dom.	
Left kidney denervated:									
Arterio-venous	0		0		12.5		25		
Cerebro	17.5		21		45		45		
Both kidneys innervated:									
Lumbal series			32				59		
Cerebro series			17.5				31		

denervation does not influence appreciably the proportion of successes, but some denervation reduces it. The failures cannot always be accounted for by the circulatory shock following decerebration. This varied very greatly from one dog to another: in some the blood pressure was hardly affected by the brain section and the flow of urine never ceased; in others, the blood pressure fell sometimes to 30 or 40 mm. Hg and could not be raised except temporarily, by any amount of intravenous infusion. Most commonly, however, in this series, the blood pressure fell to 60 or 70 mm. Hg and remained either spontaneously or after the infusion of 50-100 cc. of defibrinated blood to around 100-120 mm. Hg, where it remained.

#### SPONTANEOUS POLYURIA

The interpretation of the present series of experiments is complicated by the appearance in several preparations of spontaneous polyuria (Figs. 2 and 3). Newton and Smith [1934] occasionally observed spontaneous polyuria in hypophysectomized and decerebrate cats, but it has not been observed in any of the earlier series of this work on dogs. As is

seen in Table II, this polyuria, arbitrarily defined as a urine flow of 0.5 c.c./min. or over, is not a common phenomenon, although appearing

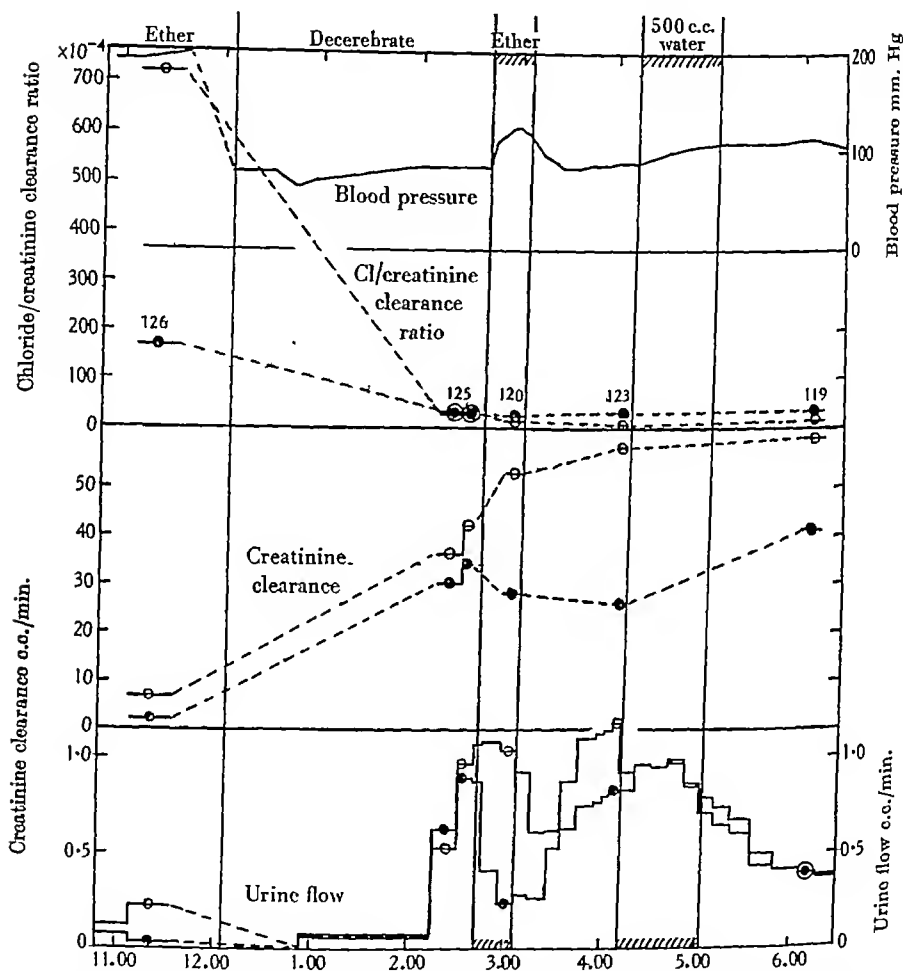


Fig. 2. Dog 23, 12 kg. Left kidney denervated 11 October 1938. Hypophysectomized and decerebrated, 25 October 1938. *Spontaneous polyuria*; checked by ether immediately in innervated, and less markedly, after considerable delay, in denervated kidney; fall in creatinine clearance in innervated, and rise in denervated kidney. Marked fall in chloride excretion on decerebration. Administration of water leads to fall in urine flow in spite of rise in blood pressure and in creatinine clearance. ○ denervated kidney; ● innervated kidney.

somewhat more frequently in preparations with one kidney denervated than in those with both kidneys innervated. It has only been observed

TABLE II

	Number of experiments showing				Total number of experiments
	Spontaneous polyuria		No spontaneous polyuria		
	Inn.	Den.	Inn.	Den.	
Left kidney denervated:					
Acute	1	2	6	5	11
Chronic	5	5	7	7	24
Both kidneys innervated:					
Edinburgh series		2	8		14

A number of experiments could not be classified. They were (a) those in which water had been given before decerebration or very shortly after, and (b) those in which the blood pressure and/or the creatinine clearance failed to rise to, and be maintained at, or above, about 80 mm. Hg and 10 c.c./min. respectively.

at blood pressures greater than about 80 mm. Hg and with creatinine clearances greater than about 10 c.c./min., and when these values are maintained for an hour or so.

Spontaneous polyuria in these preparations is closely related to water diuresis. Neither appears unless there is an adequate blood pressure and creatinine clearance. Both are checked by ether anaesthesia and by the injection of pituitary extracts. In both, the inhibition by pituitary extracts takes place with a slight rise in creatinine clearance. In both the inhibition by ether is less marked, and may be absent, in the denervated kidney, and is accompanied by a fall in creatinine clearance (Table V). It is possible, indeed, that some of the apparently spontaneous polyurias were, in fact, the result of a water load existing in the dogs before decerebration, but we have no evidence of this. The dogs were allowed access to water before the operation, but none was given deliberately. None of the chronically denervated animals showed gross polyuria or polydipsia before decerebration.

The question arises, therefore, as to whether the increase in urine flow that follows the administration of water is not due to a release of a spontaneous polyuria previously latent owing to an inadequate glomerular filtration rate. The absorption of a relatively large volume of water would be expected to result in a rise of blood pressure, possibly in a reduction of vaso-motor tone, and in an appreciable dilution of the blood. If this were so, some doubt would arise as to whether the response to water in the hypophysectomized and decerebrate preparation was in any way comparable to that in the intact animal. It might be held that removal of the hypophysis and the hypothalamic centres always resulted in an

essentially polyuric preparation, and no mechanisms for the control of water diuresis, other than those already postulated, need be looked for.

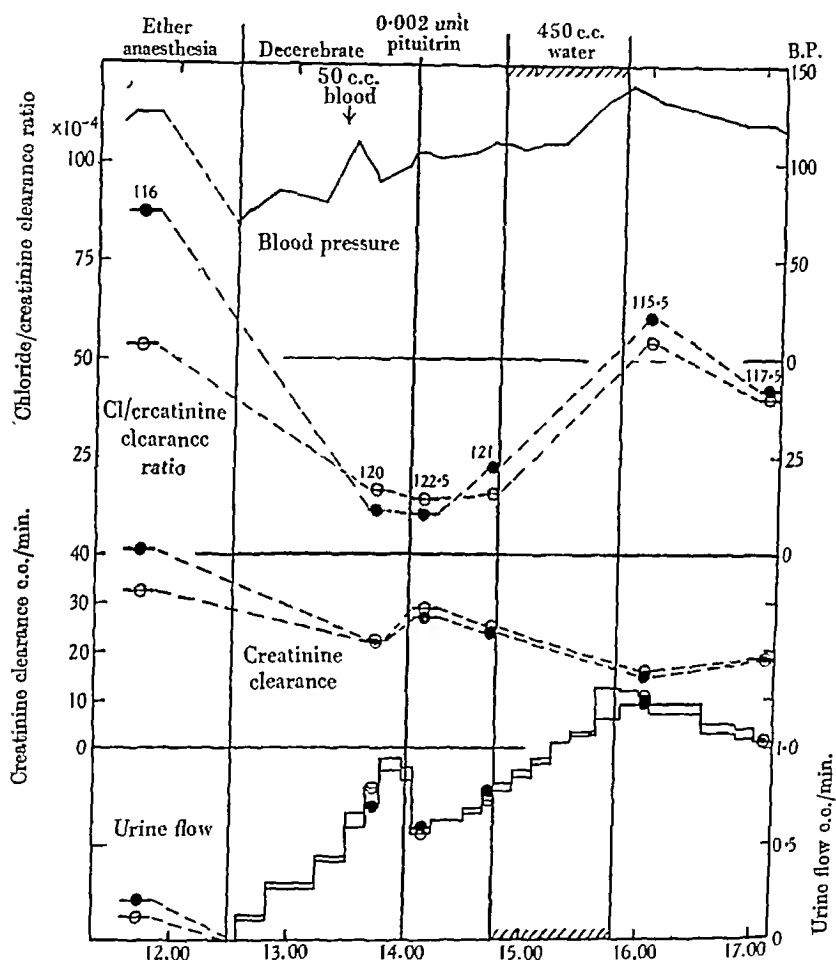


Fig. 3. Dog 34, 12.5 kg. Both kidneys innervated. *Spontaneous polyuria*; checked by 0.002 unit pituitrin (P.D. and Co.) with slight rise in creatinine clearance. Doubtful response to water. ○ left kidney; ● right kidney.

Several observations, however, are inconsistent with this hypothesis. (a) It is possible to get a response to water in preparations which fail to develop a spontaneous polyuria in circumstances at least as favourable as those in which other preparations do develop one. Thus Exps. 34 and 35 may be compared (Figs. 3 and 4). In Exp. 34 a spontaneous polyuria

developed within 1 hr. of decerebration, the blood pressure rising from about 75 mm. Hg to about 100 mm. Hg, and the creatinine clearance rising

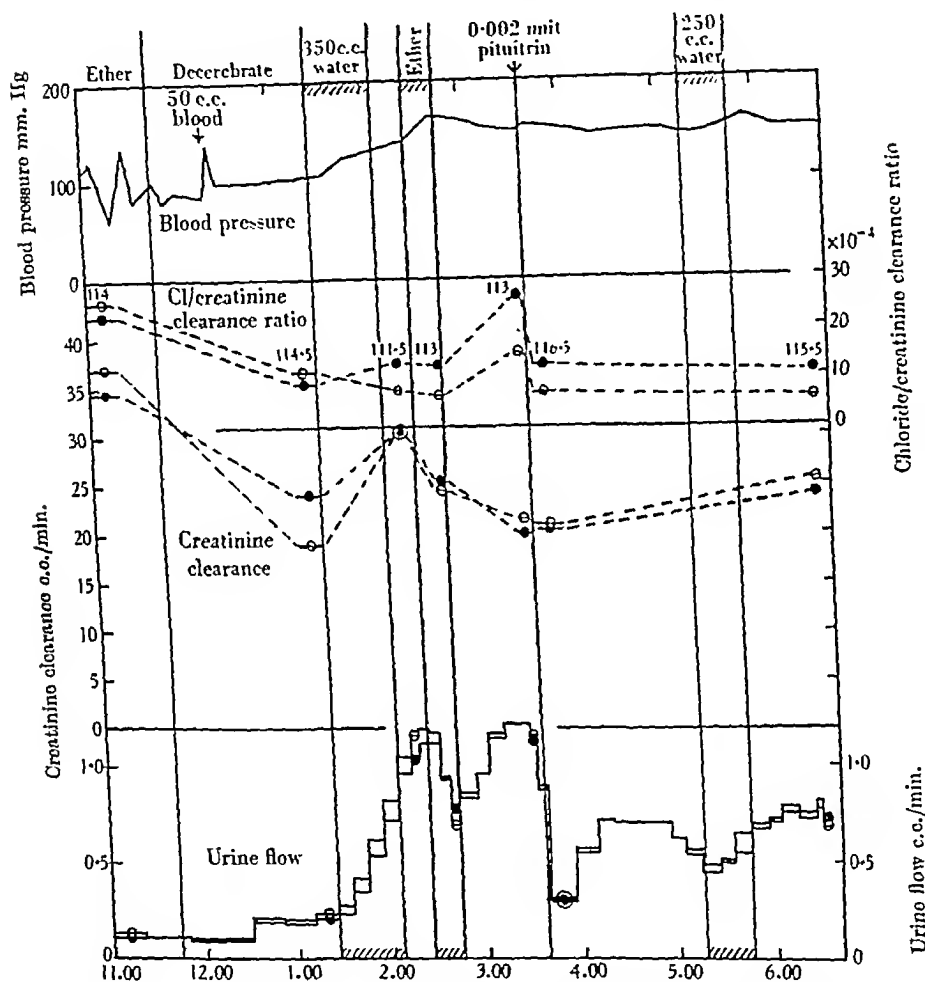


Fig. 4. Dog 35, 11 kg. Both kidneys innervated. Water diuresis, with transient rise in creatinine clearance; checked by ether, with fall in creatinine clearance, and by 0.002 unit pituitrin (P.D. and Co.) without change in creatinine clearance. A second administration of water leads to a second water diuresis. ○ left kidney; ● right kidney.

to 22 c.c./min. In Exp. 35, no spontaneous polyuria developed, even though the blood pressure was 90–100 mm. Hg for 1½ hr. after decerebration, and the creatinine clearance reached 18 and 24 c.c./min. in the two kidneys respectively. Administration of water resulted in a marked



polyuria. (b) It is sometimes possible to obtain two responses to the administration of water on the same preparation (Fig. 4). In this series we have been able to observe this in two dogs each with one kidney denervated (in one, only the denervated kidney responded the second time) and in one dog with both kidneys innervated. In none of these was there any evidence that the falling off of the first response was due to an inadequate blood pressure or creatinine clearance. Newton and Smirk [1934] have called attention to the importance of being able to obtain two responses to water in the hypophysectomized and decerebrate preparation, as evidence for the existence of some control over diuresis other than that due to the pituitary body. (c) In some preparations a response to water was superimposed on a spontaneous polyuria, without more than a transient rise in blood pressure or creatinine clearance. On the whole, then, our observations confirm those of Fee [1929] and of Newton and Smirk [1934]; hypophysectomy and decerebration do not necessarily lead to polyuria.

#### THE CONTROL OF WATER DIURESIS

Fee [1929] has recorded the appearance of polyuria in the denervated kidney after hypophysectomy and decerebration, while the urine flow on the innervated side was normal. On the basis of this, and of the results obtained by Bayliss & Fee [1930] on the effect of denervation in increasing the urine flow in the perfused innervated kidney, it was predicted that hypophysectomy would always lead to polyuria in a denervated kidney. Polyuria, in fact, could be prevented either by the presence of the pituitary body or by the action of the renal nerves. We, however, have always observed polyuria, if at all, in both the innervated and the denervated kidney. This clearly complicates the problem and suggests that the renal nerves are not responsible for its inhibition except, possibly, as a result of simple vasoconstriction and reduction in glomerular pressure. Taking into account the observations of Verney [1929] and of many others, who have shown that simple hypophysectomy does not, in acute experiments, under anaesthesia, necessarily lead to polyuria, it would appear to be necessary to postulate some diuretic or anti-diuretic hormone, in addition to those supposed to be derived from the anterior and posterior lobes of the pituitary body.

#### CORRELATION OF THE TYPE OF RESPONSE WITH THE LEVEL OF BRAIN SECTION

At the end of each experiment post-mortem examination was made in order to discover as accurately as possible the exact position at which

the brain stem had been divided. For conciseness in presenting the results, each level has been given a number and letter, as indicated in Table III, and from the Table it will be seen that there appears to be no

TABLE III. Correlation with level of brain section

Polyuria			No polyuria	
Spontaneous	After water		Low blood pressure	Other reasons
	Certain	Doubtful		
1a <sup>1</sup>	1a <sup>1</sup>	2a	1a <sup>1</sup>	1a <sup>1</sup>
2a <sup>2</sup>	2a	2c	2a	2a
2b	3a	3a	2b	2c
3b <sup>2</sup>	4b	3b	2c	3b
4a <sup>3</sup>	5b	3c	3a	4a
4c <sup>3</sup>		4a	3c	4b
5b		5b	4a	5b
		5c	4b	
			5a	

<sup>1</sup> Pituitary intact.

<sup>2</sup> Pituitary intact but stalk severed.

<sup>3</sup> Polyuria ceased; no subsequent response to water.

#### Explanation of notation used

Ventral: 1, anterior to optic chiasma.

2, middle of optic chiasma.

3, middle of tuber cinereum.

4, posterior margin of tuber cinereum.

5, level of IIIrd nerves, or more posterior.

Dorsal: a, anterior margin of superior corpora quadrigemina.

b, middle of ditto.

c, posterior margin of ditto, or more posterior.

correlation between the presence or absence of polyuria, or of a response to water, with the position of the brain section. We are at a loss to explain this observation. It may be that the hypothetical diuretic centre has different anatomical relationships in different dogs, and in this connexion it will be remembered that Reichert & Dandy [1936] were unable to find any relation between the exact position of a puncture in the hypothalamic region and the presence or absence of polyuria subsequently. It may be, also, that the *physiological* level of the section differs appreciably from the anatomical level, and that the difference varies from one dog to another. One might expect, for example, that where there had been much haemorrhage during the operation of hypophysectomy and decerebration, the physiological level of the section would be more removed from the anatomical level than it would if the haemorrhage had been slight and well controlled; we can, however, obtain no evidence for this from our

observations. Alternatively, of course, it may be that the structure responsible for the onset of polyuria is not located in the hypothalamus, and that its activity is controlled by totally unrecognized factors.

#### THE ACTION OF ANAESTHETICS

Fee [1928] has shown that a true water diuresis cannot be obtained in dogs under the anaesthetics ether, chloroform, paraldehyde, urethane or amytal, or under morphia. Newton & Smirk [1933], on the other hand, obtained water diuresis in cats under chloralose; it was inhibited by ether and chloroform. Theobald [1934] obtained it in dogs asleep under chloralose, but not fully anaesthetized. The action of ether has been repeatedly studied in this series of experiments, and a few observations have been made on the action of nembutal and chloralose. We have confirmed the fact that before hypophysectomy and decerebration, the administration of water to dogs under ether has no appreciable effect on the urine flow. After hypophysectomy and decerebration, and after the ether has blown off, the administration of ether during a polyuria, either spontaneous or after the administration of water, instantaneously reduces the urine flow in the innervated kidney, but has only a smaller, and often delayed, action on the denervated kidney. The creatinine clearance of the innervated kidney is always reduced, but that of the denervated kidney follows the change in blood pressure, which may rise, fall or remain unaffected. The mean change in urine flow and creatinine clearance in the two kidneys as a result of the administration of ether six times to five dogs is shown in Table IV. It would appear, therefore,

TABLE IV. Inhibition of polyuria

Values expressed as percentages of value just previous to inhibition (geometric means).

	By ether		By pituitary extract
	Den.	Inn.	
Urine flow	$58 \pm 15$ (6)	$22 \pm 4$ (6)	$48 \pm 9$ (6)
Creatinine clearance	$96 \pm 12$ (6)	$75 \pm 6$ (6)	$113 \pm 5$ (6)
Urine chloride concentration	$171 \pm 43$ (6)	$171 \pm 46$ (6)	$172 \pm 8$ (6)
Chloride/creatinine clearance ratio	$98 \pm 13$ (6)	$46 \pm 6$ (6)	$71 \pm 10$ (6)

that the chief action of ether is by way of the renal nerves, bringing about in all probability, vasoconstriction and a fall in glomerular pressure; the smaller and delayed action on the denervated kidney may in part be due to a release of adrenaline. The matter, however, is not quite so simple as this, since the creatinine clearance not infrequently fails to return to its initial value as the ether blows off, even though the urine flow does.

Nembutal was given to one animal only, during a water diuresis, with the result that the urine flow from the innervated kidney was markedly reduced, while that from the denervated kidney remained unaffected. No measurements were made of the creatinine clearance.

Chloralose, however, appeared to have a smaller action on both the creatinine clearance and the urine flow in innervated kidneys than had ether, but only a few experiments have been performed. It is possible, therefore, that a true water diuresis might be obtained in a hypophysectomized and decerebrate dog under chloralose, although we have not yet succeeded in so doing.

The action of ether may be contrasted with that of pituitary extracts (Figs. 1 and 2 with Fig. 3; Fig. 4; Table IV). The latter not only fails to lower the creatinine clearance in either the innervated or the denervated kidney, but usually raises it slightly [confirming Samaan, 1935]; the urine flow is reduced equally in both kidneys. Moreover, a saline diuresis, produced, for example, by the intravenous administration of 4 g. NaCl in 60 c.c. water, after the dog failed to respond to the administration of 200 c.c. water, was inhibited by ether, with the usual fall in creatinine clearance, but was unaffected by pituitary extract. These observations confirm, and extend, those of earlier workers, both in the absence of any marked action of pituitary extracts on the rate of glomerular filtration, and on the lack of inhibitory action on a diuresis produced by concentrated saline solutions [cf. Smith, 1937].

It must be emphasized that the relative absence of inhibitory action by ether on the denervated kidney applies only to the hypophysectomized and decerebrate preparation. We have never obtained any suggestion of a water diuresis, even in the denervated kidney, in the intact animal under ether or chloralose. It is possible that if the pituitary body is intact, administration of anaesthetics prevents the inhibition of its secretion that is supposed ordinarily to follow the administration of water. Anaesthetics, in fact, result in an inhibition of inhibition of the pituitary inhibition! It may be recalled, in this connexion, that Shen [1937] has observed that anaesthetics prevent the inhibition of the secretion of the melanophore expanding principle of the posterior pituitary body in the frog.

#### THE EXCRETION OF CHLORIDES

The chloride concentration of the urine of our hypophysectomized and decerebrate preparations was often extremely low. This was not due to the rate of urine flow being abnormally high in relation to the rate of glomerular filtration, since even when this factor was taken into account,

and the results expressed as the chloride/creatinine clearance ratio, the values obtained after hypophysectomy and decerebration were often very much lower than they had been before. (This is well shown in Figs. 1 and 2.) There was no correlation with the changes in the chloride concentration of the blood, which in any case were mainly small, there being a rise of up to 5% in five experiments, a fall of up to 4% in three and no change in one. The rate of excretion of chloride rose in three of these experiments and fell in six. The presence or absence of a polyuria, moreover, made no appreciable difference to the concentration of chloride in the urine, with the result that, as is seen in Table V, the chloride/creatinine clearance ratio tends to be higher in the presence of a spontaneous polyuria than in its absence. The average results for all the

TABLE V. The effect of decerebration on the chloride/creatinine clearance ratio. (All figures to be multiplied by  $10^{-4}$ .)

	Denervated series		Innervated series	
	Den.	Inn.	Left	Right
Under ether	$128 \pm 99$ (7)	$46 \pm 21$ (7)	$32 \pm 9$ (5)	$36 \pm 13$ (5)
Decerebrate: no polyuria	$15 \pm 5.5$ (6)	$14 \pm 5.5$ (6)	$14 \pm 3.7$ (10)	$9 \pm 3.0$ (10)
Decerebrate: spontaneous polyuria	$38 \pm 11$ (5)	$30 \pm 2.5$ (5)	$28 \pm 12$ (2)	$19 \pm 7$ (2)

experiments in which figures are available are shown in Table V. Detailed examination of the individual experiments from which this Table was constructed shows that they fall roughly into two classes: (a) those in which the chloride/creatinine clearance ratio was very markedly reduced (to  $\frac{1}{2}$ – $\frac{1}{3}$ ), and (b) those in which it remained sensibly constant or rose somewhat. Out of eleven experiments, four were in class (b), but one of these may be discounted, as the rate of chloride excretion was very small even before decerebration. Of the ten remaining experiments, in two the tuber cinereum was untouched, and one of these fell in class (b); in six experiments, the section passed through the tuber and two were in class (b); in two experiments, the tuber was entirely removed, and neither was in class (b). The number of experiments is clearly too small for any definite conclusion to be reached, but there seems to be a suggestion that some structure in the tuber cinereum is concerned with the excretion of chloride by the kidney.

The effect of the *administration of water* on the chloride concentration of the urine depended upon whether a polyuria resulted or not. As is shown in Table VI, the onset of a polyuria was almost always associated with a fall in the chloride concentration of the urine, whereas if there was

TABLE VI. Effect of water administration on chloride excretion

	Diuresis		No diuresis
	Certain	Doubtful	
Number of experiments in which:			
Urine chloride concentration			
Rose	0	2	13
Was unchanged	1	0	1
Fell	11	5	1
Chloride/creatinine clearance ratio			
Rose	6	2	8
Was unchanged	0	0	0
Fell	6	3	5

no polyuria the urine flow often decreased (Fig. 2) and the chloride concentration rose. These changes were the result mainly, if not solely, of the changes in the rate of urine flow, as is shown by the inconstant behaviour of the chloride/creatinine clearance ratio; this gives no indication that the administration of water has any consistent effect on the fraction of the filtered chloride which is reabsorbed.

Inhibition of a polyuria, either spontaneous or after the administration of water, either by ether or by pituitary extracts, was associated with a rise in the chloride concentration of the urine. As is shown in Table IV, however, the chloride/creatinine clearance ratio remains unchanged in the denervated kidney during ether administration, and falls markedly in the innervated kidney. There is no reason to suppose that this fall is not due merely to the fall in creatinine clearance. Pituitary extracts, however, result in a slight fall in the chloride/creatinine clearance ratio, in spite of the rise in the creatinine clearance; this would seem to imply that pituitary extracts increase the fraction of the filtered chloride which is reabsorbed. The chloride clearance, however, uncorrected for the changes in creatinine clearance, showed no significant change, in agreement with the observations of White & Findley [1939], who observed no increase in chloride excretion in man after the administration of 0.15 unit of pitressin per kg. Previous workers on the whole, however, have observed an increase in the excretion of chloride after the administration of pituitary extracts, although the changes have often been slight and inconstant [Smith, 1937].

#### DISCUSSION

Briefly, the present conception as to the mechanisms controlling the excretion of water appear to be: (1) that the posterior part of the pituitary body elaborates an anti-diuretic factor at a rate which is, or may be, controlled by impulses passing from the supra-optic, and possibly other, nuclei in the anterior hypothalamus [Ranson, 1936]; (2) that the removal

of this anti-diuretic factor will only lead to a polyuria if a diuretic factor is present, the elaboration of this factor being controlled by the anterior part of the pituitary body [Ranson, 1936]; (3) that the anterior pituitary probably does not itself secrete the diuretic factor, but only controls its secretion by the thyroid [Mahoney & Sheehan, 1936; White & Heinbecker, 1937; Biggart & Alexander, 1939] or the adrenal cortex [Silvette & Britton, 1938]. Spontaneous polyuria (diabetes insipidus) is produced by the interruption of the nerve tracts controlling the activity of the posterior pituitary, or removal of the posterior pituitary. Complete removal of the whole pituitary body does not uniformly result in polyuria, owing to the simultaneous removal of both anti-diuretic and diuretic factors, but does so, presumably, only when the spontaneous activity of the thyroid or the adrenal cortex, as the case may be, is great enough. Administration of water produces diuresis as a result of inhibition of the posterior pituitary, possibly by way of the nerve tracts supplying it.

When we come to apply these conceptions to our own observations, we meet with certain difficulties. We have removed the whole of the anterior hypothalamus, and both anterior and posterior lobes of the pituitary, so that none of the hypothalamic or hypophyseal mechanisms just mentioned can have been operative. Accessory anti-diuretic regions, e.g. the tuber cinereum and the mamillary bodies, have been postulated, but in many of our experiments, as in those of Newton & Smirk [1934], all these regions have been removed, without the production of polyuria. In view of this, we have felt more inclined to postulate the existence of a diuretic factor, but the lack of correlation between the position of the brain section and the occurrence of polyuria raises a doubt as to whether its elaboration is controlled by any structure in the brain stem. On the basis of the previous work, it would seem to be at least related to the activity of the thyroid or the adrenal cortex. The irregular occurrence of spontaneous polyuria would have to be accounted for by assuming a greater activity of the thyroid or adrenal cortex in some of our dogs than in the others. It would appear, also, to be necessary to postulate a direct action of the ingested water on the organ or gland responsible for the formation of the diuretic factor. The rapidity of the response, however, makes it rather unlikely that the diuresis results solely from an increased secretion by the thyroid, and, while the observations of Silvette & Britton [1938] on the action of the adrenal cortex in opossums seems definite enough, they have not been confirmed in dogs; indeed Biggart & Alexander [1939] failed to observe any action of cortical extracts on their puncture polyurias, whereas administration of thyroid increased the diuresis. The

polyuria that they observed immediately after the puncture, however, differed from ours in being unaffected by posterior pituitary extracts.

Such an hypothesis, moreover, does not provide a satisfactory explanation of why some of our preparations responded to the administration of water, and others did not. We have no reason to suppose that the hypothetical mechanisms were not functioning normally before hypophysectomy and decerebration; and to suppose that the division of the brain stem interfered with the operations of the thyroid or adrenal cortex in some preparations more than in others, brings us back to the lack of correlation between the behaviour of the preparation and the level of the brain section. Individual differences between the dogs appears to be the unsatisfactory, but only possible, explanation.

### SUMMARY

1. The renal nerves play no part in the production of water diuresis in the hypophysectomized and decerebrate dog.

2. Spontaneous polyuria sometimes follows hypophysectomy and decerebration. The renal nerves play no part in its onset.

3. There appears to be no correlation between the position at which the brain stem is divided and (a) the occurrence of spontaneous polyuria, or (b) the capability of yielding a water diuresis.

4. Inhibition of spontaneous polyuria and water diuresis by ether is chiefly the result of a fall in creatinine clearance, produced by the action of the renal nerves. Inhibition by pituitary extracts takes place without change in creatinine clearance, and the renal nerves play no part (confirming earlier workers).

5. Many, but not all, the preparations show a marked fall in chloride excretion after hypophysectomy and decerebration. There is a suggestion that this only occurs if the tuber cinereum is damaged or removed.

6. The excretion of water appears to be independent of that of chlorides.

7. None of the existing theories of the control of water excretion appears to be entirely capable of accounting for all our observations.



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THE TIME COURSE OF THE OXYGEN CONSUMPTION  
OF STIMULATED FROG'S MUSCLE

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STIMULATION of an isolated muscle in oxygen causes a temporary increase in its rate of oxygen consumption. It is of interest to inquire whether this extra oxygen consumption occurs as an immediate reaction to excitation, the oxidative process providing the energy for contraction, or whether the extra oxygen consumption takes place as a recovery process afterwards. Myothermic experiments seem unequivocally in favour of oxidation being a recovery process. Direct methods have led to conflicting results. The only attempts of any importance have been described by Fenn [1927] and Millikan [1937]. The inconclusiveness of their results depended (1), in Millikan's method, on the impossibility of distinguishing between "initial" and "recovery" processes in a muscle with a rate of metabolism as high as that of a cat's soleus at 37° C., and (2), in Fenn's method, on the impossibility of making appropriate corrections for the slowness of diffusion of oxygen through the muscle and of the evolution of carbon dioxide.

The present method of determining the time course of oxygen consumption of stimulated muscle was designed to obviate these two important sources of ambiguity. The confusion of initial and recovery processes is rendered unimportant by using a short duration of stimulus with a frog muscle cooled to 0° C. The methods of dealing with the diffusion of oxygen and carbon dioxide will be considered later.

## METHOD

*Apparatus.* An isolated pair of sartorius muscles of an English frog (*Rana temporaria*) is used. The muscles are balanced against each other in the two bulbs of a differential volumeter (Fig. 1). This volumeter is exactly similar, in principle, to that used by Schmitt [1933] for investigations of the metabolism of nerve. Each muscle is supported in the gas

space on a glass frame which is part of the stopper. The tibial end of the muscle is slipped through the ring at the upper end of the frame. Part of the pelvic bone is left attached to the muscle and this rests on the upper surface of the ring. A thread, tied to the tibial end of the muscle, is attached to the lower end of the frame. The muscle is stimulated by platinum electrodes, the external leads to which are protected by narrow rubber tubing. The capillary tube between the bulbs has an internal diameter of 0.39 mm. or an area of cross-section of 0.11 mm.<sup>2</sup> A drop of petroleum ether (b.p.

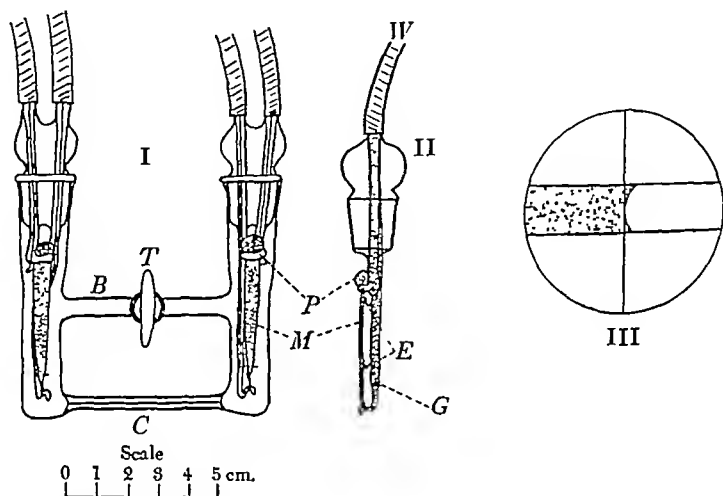


Fig. 1. Differential volumeter. I, with stoppers and muscles in position. II, stopper with muscle. III, capillary and index drop as seen by microscope with cross-wire in position of reference. Diameter of capillary 0.39 mm. *M*, sartorius muscle; *P*, pelvic bone; *E*, stimulating electrodes; *G*, glass support; *W*, rubber tube insulating leads; *B*, bridge, with tap, *T*; *C*, capillary tube.

greater than 140° C.) about 2 mm. long is used as indicator in this tube: it is introduced on the end of a piece of copper wire (20 s.w.g.). The bridge, with stopcock, joining the two bulbs enables the position of the index drop to be adjusted and is essential when placing the stoppers in position and during the period of temperature equilibration. Since the capillary tube is short and does not allow much unilateral volume change, the stopcock is turned off only shortly before readings are to be taken.

The carbon dioxide produced during rest and activity is absorbed by soda placed on a strip of filter paper which is stuck to the wall of the vessel. The soda used is isotonic with Ringer's solution (0.125 *M*), in order to avoid transference of water by distillation from the muscle to the soda

which would occur if the soda were stronger. The muscle remains excitable much longer if drying is thus prevented. It is easily shown that there is sufficient soda present to deal with the carbon dioxide produced during the period of the experiment. The adequacy of this method of absorption of carbon dioxide will be discussed later.

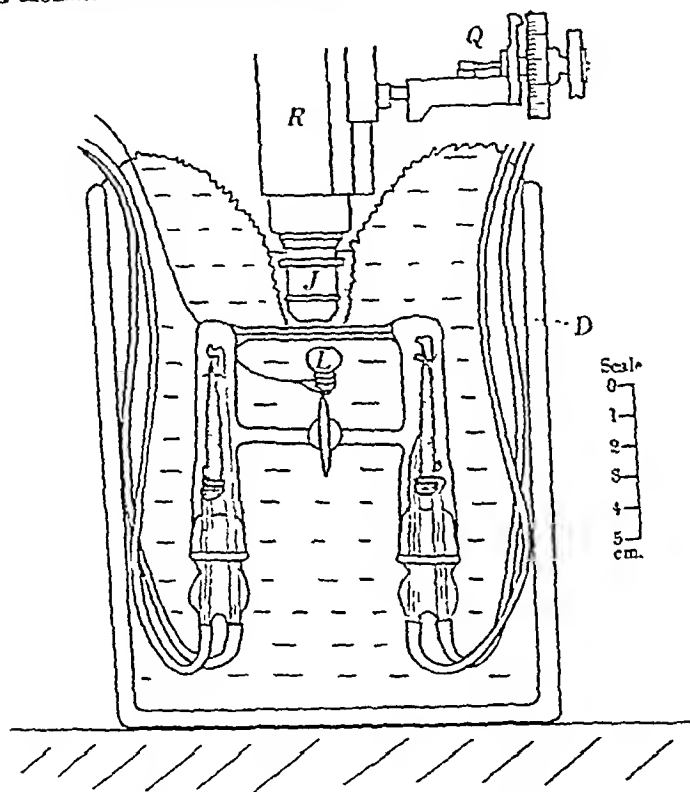


Fig. 2. Volumeter in ice. *D*, wall of Dewar flask; *L*, flash-lamp bulb; *J*, objective of microscope lowered into water; *R*, tube of microscope; *Q*, micrometer screw.

The whole volumeter is immersed, capillary tube uppermost, in a Dewar flask full of finely crushed ice and a small quantity of water (Fig. 2). The ice is cleared away in a small area over the index drop and the objective of a travelling microscope is lowered into the water. The index is brightly illuminated by means of a flash-lamp bulb, soldered to two wires, which is pushed down into the ice. The lamp is turned on only when required to take readings, as the heat it produces, if left on, may cause movement of the index. Readings are taken with the cross wire of the microscope perpendicular to the capillary and tangential to the meniscus of the drop. The

magnification is 20 diameters. The microscope is shifted by a micrometer screw, graduated to  $\frac{1}{200}$  mm., and this is the smallest movement recorded: it corresponds to a unilateral volume change of about  $10^{-3}$  mm.<sup>3</sup>

Besides keeping the muscle at 0° C. and so facilitating the resolution of the different metabolic phases, the mixture of ice and water provides a very necessary and accurate thermostat. The volume of each bulb of the volumeter is as small as possible, about 5 c.c. The sensitivity is independent of the volume, but the pressure driving the index, for a given total volume change, is increased, and the effect of unilateral temperature change is reduced, if the volume of the bulb is kept low. Even with a bulb volume as small as 5 c.c. a unilateral temperature change of  $10^{-3}$  °C. would cause a movement of the index of 0.1 mm. and a serious disturbance: hence the necessity for a good thermostat. This can only be obtained by surrounding the volumeter with finely crushed ice with a *small* amount of water. A half-and-half mixture of ice and water kept well stirred by bubbling air is unsatisfactory. The net change of temperature is nil, but rapid to-and-fro movements of the index occur, indicating temperature pockets.

*Cleaning the capillary tube.* It is very important that the capillary tube be extremely clean in order that the index shall move smoothly over the very small distances recorded. This is particularly necessary in the early stages when, as will be seen later, there is an initial very small increase in volume due to heat production. When the tube has not been properly cleaned, this heat expansion may be under-registered or even not observed at all. In this case, apart from the impossibility of accurate analysis of the result, the observer has no immediate sign of activity of the muscle and cannot gauge the magnitude of the response. Similarly, in the later stages of the oxygen uptake a stickiness in the tube will render doubtful the shape and final maximum of the record. The method adopted for cleaning is to immerse the volumeter, containing dichromate-sulphuric acid, for several hours in boiling water. The tube is then washed with distilled water, alcohol and finally anaesthetic ether.

It might be thought to be advantageous to increase the sensitivity of the volumeter by using a capillary tube of narrower bore. That is not the case: there is a minimum size of tube that can be kept free from all traces of stickiness. The word "stickiness" refers here to "stick-and-slip" friction as distinct from viscous drag. By adequate cleaning it can be eliminated completely in a tube not too small, whereas the viscous drag must always remain and be a function partly of the size of the tube. The presence, however, of viscous drag is of no importance and there is, therefore, no advantage to be gained by using a tube larger than that which can be kept free from stickiness. The limit is set by the pitch of the micrometer screw. Actually, in a number of experiments in which the maximum sensitivity was not required a larger tube, of bore 0.55 mm., was used. It is much easier to keep clean.

In the paper by Schmitt [1933] can be found a full account of the considerations involved in the choice of fluid for the index. Dimethyl aniline, butyric acid and valerianic acid have been tried, but are less satisfactory than petroleum ether (b.p. greater than  $140^{\circ}$  C.) owing to their higher vapour pressure.

*Base drift.* Unless certain precautions are taken, it is found that a slow drift of the index remains after the necessary time for temperature equilibration has elapsed. Control experiments made with the muscles absent show that this cannot be accounted for by inequality in the rate of resting oxygen consumption of the two muscles. Schmitt [1933, 1936] has made a study of the causes of base drift; these are:

(1) *Slipping at ground joints.* The area of cross-section of the stopper is about  $2\text{ cm.}^2$  and a movement of  $0.01\text{ }\mu\text{/min.}$  will cause a drift of the index as great as that produced by working non-differentially. This slipping can be prevented by using vaseline as the joint lubricant: the stopper is then turned in its seating until it jams.

(2) *Unequal evaporation at the two surfaces of the index.* This may be caused by a previous unidirectional movement of the index with consequent wetting and increase of the area for evaporation on one side. The effect can be minimized by running the index up and down the capillary tube several times before the bridge is finally closed.

By jamming the stoppers and wetting the walls of the capillary before closing the bridge, it has been found possible to diminish the time necessary for equilibration to half an hour or even less.

*Stimulus.* The muscles are stimulated by a thyatron circuit at a frequency of about 5 per sec. The frequency is kept to the minimum required to maintain a tetanus. By doing this, and also by designing the thyatron circuit to give excitation with least stimulus energy ( $RC$  of discharge circuit equal to the time constant of excitation of the muscle), the heating effect of the stimulus can be made negligible. Polarization is avoided by both charging and discharging the condenser through the muscle.

*Control heating.* Artificial heating of the muscle without stimulation is needed in order to make a control curve to correct for heat evolved during muscular activity. This is provided by high-frequency alternating current ( $10^5$  c./sec.) passed between the stimulating electrodes. The muscle is not excited by a current of this frequency.

*Treatment of the muscles.* The muscles, mounted on the frames, are soaked in a cold store in oxygenated Ringer's solution containing phosphate buffer at pH 7.2 (10 mg. P/100 c.c.). Muscles which are soaked for not less than 12 hr. remain excitable for many hours in a gas space at  $0^{\circ}$  C.

*Supply of oxygen to the muscles.* The bulbs of the volumeter are filled with oxygen from a cylinder. To reduce the effect of slipping at ground joints there are no side tubes and the oxygen is introduced through a tube to the bottom of the open bulb, expelling the air. The stopper is then inserted. The exact composition of the gas in the chambers is unknown, but this does not matter: the amount of air mixed with the oxygen is certainly small. The adequacy of supply of oxygen to the muscle at rest and following stimulation must be considered:

(1) *At rest.* It can be calculated [Hill, 1928] that for a strip of muscle at  $0^{\circ}\text{C}$ ., exposed on both sides to pure oxygen, the greatest thickness which can be fully supplied with oxygen is 9.4 mm. The average thickness of the sartorii used was only 0.9 mm.

(2) *Following activity.* The time course of oxygen consumption of stimulated muscle must not, at this stage, be presumed. We must admit the possibility that the excess oxygen usage associated with activity coincides with contraction. If, with this assumption, the oxygen already present in solution is adequate, then a delayed oxygen consumption following activity cannot be due to oxidation of metabolites produced only in the absence of oxygen. It can be calculated that at  $0^{\circ}\text{C}$  the concentration of oxygen in the innermost layer of a resting muscle 1 mm. thick exposed on both sides to oxygen is  $38\text{ mm.}^3\text{ O}_2/\text{c.c.}$  In the experiments to be described the greatest duration of tetanus employed was 12 sec. The total quantity of oxygen required was  $26\text{ mm.}^3\text{ O}_2/\text{c.c.}$  There could therefore have been no lack of oxygen in any part of the stimulated muscle. If the oxygen consumption proves in fact to follow a slow recovery process, then assurance is made doubly sure by further diffusion of oxygen.

## RESULTS

When the index is stationary and equilibration is complete, one muscle (*A*) is stimulated for 12 sec. During the stimulus there is a volume increase (Fig. 3) which ceases when the stimulus ends. The volume then decreases, reaches its original value in about  $1\frac{1}{2}$  min., and then continues to decrease for about 30 min., when the index comes finally to rest (Fig. 4). Deflexions are recorded from the micrometer screw and times are taken with a stopwatch. The whole process is then repeated with the other muscle (*B*). When the oxygen consumption is complete, *B* is stimulated again: then *A* twice: then *B* twice, and so on, finishing with *A* once. The records (an equal number taken with either muscle) are then added together. By stimulating the muscles in this sequence a small variable base drift is approximately compensated.

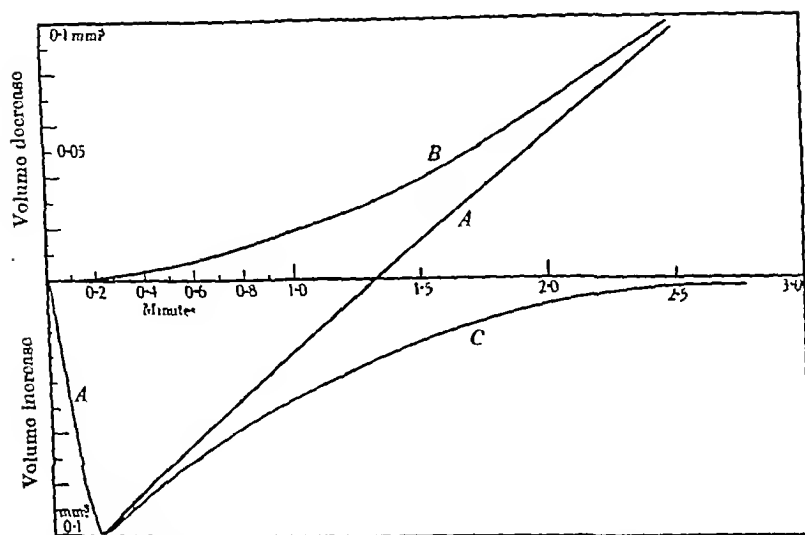


Fig. 3. *A*, early stages of record on large scale. *C*, control heating scaled to same maximum. *B*, record corrected for initial heat production of muscle.

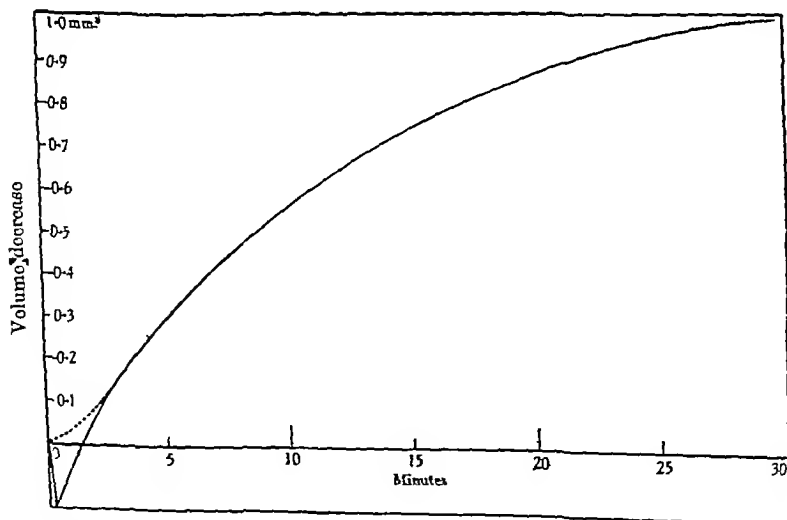


Fig. 4. Complete record of movement of index following a 12 sec. tetanus. Portion corrected for heat is dotted. Mass of muscle about 100 mg. Thickness about 0.9 mm.



In order to find the time course of the oxygen consumption of the muscle cells, an analysis is necessary in which three factors must be considered:

- (1) The heat production of the muscle.
- (2) The carbon dioxide production of the muscle.
- (3) The rate of diffusion of oxygen into the muscle.

*The heat production.* The initial volume increase is of the magnitude to be expected from the initial heat production. (The size of the latter can be calculated later from the total oxygen consumption, assuming that  $(\text{initial heat})/(\text{recovery heat}) = \frac{1}{2}$ .) To show this, it is necessary to make a heat calibration. The muscle is made inexcitable by soaking in isotonic potassium chloride and is then heated by a known condenser discharge. The index reaches its final position very rapidly (within a second) and the heat is then slowly dissipated. In a tetanus as long as 12 sec. it is sufficient to assume that the initial heat is given off in equal "blocks" in equal intervals of time during the stimulus. Each "block" would cause a movement of the index similar to that produced by "instantaneous" heating, and it is possible to build up a calculated curve, to which the initial heat, in the absence of gaseous exchange, would be expected to give rise. When this is done, the values of the observed and calculated maxima are very nearly the same. The initial expansion, therefore, is large enough to represent the heat production of the muscle, so that oxygen uptake from the gas is of no importance before the end of the 12 sec. stimulation.

Heat calibration is not done by means of the high-frequency oscillator, since it would be difficult to express the energy output into the muscle in absolute units.

For a tetanus as long as 12 sec. it can be assumed that the rate of initial heat production is constant, and to correct for it a "heating control" is required. This is obtained by heating the muscle artificially with the high-frequency oscillator for the same duration as the tetanus. The movement of the index during and after the heating is observed. This "heating control" is scaled down to the same maximum as the heat deflexion of the "live" curve and is then subtracted from the recorded numbers. The record is thus corrected for the initial heat production (Fig. 3). The recovery heat, equal in magnitude to the initial heat [A. V. Hill, 1939], is produced so slowly that it requires no correction.

*Absorption of carbon dioxide.* The carbon dioxide produced must finally be absorbed by the soda on the wall of the vessel. It might be thought that the delay in passage of carbon dioxide from the surface of the muscle to the soda on the wall would cause a complication, especially in the early

stages when the rate of production of carbon dioxide is changing most rapidly. In these important early stages, however, any carbon dioxide produced is retained in the muscle which has become alkaline by the breakdown of phosphocreatine. This change towards alkalinity occurs at all hydrogen-ion concentrations between pH 3 and 8 [Meyerhof & Lohmann, 1928; Lipmann & Meyerhof, 1930]. The change is large: at pH 6 it has a maximum and the breakdown of one molecule of phosphocreatine results in an alkalinity equivalent to one molecule of NaOH.

In order to show that the carbon dioxide really is being retained in the early stages after the stimulus, the soda is omitted from the chamber. It is then found that, after the initial expansion, the rate of volume decrease is actually *greater than before*. The reason is that the muscle is reabsorbing from the gas space the carbon dioxide which it had given out during the period of equilibration. The rate of volume decrease soon falls off, and as the phosphocreatine is resynthesized the carbon dioxide is given off into the gas space again, and the index finally returns nearly to its original base line, since the respiratory quotient is about 1.

It is only in the later stages, therefore, that the problem of diffusion of carbon dioxide has to be considered. For the theoretical solution the author is much indebted to Mr P. E. Marrack: it is complicated and is not included here. It shows that for instantaneous liberation of carbon dioxide in the muscle an appreciable quantity will, for a short period, be present in the gas space. But we are more concerned here with the case of continuous production; the amount present in the gas then rapidly becomes negligible compared with the total produced. Experimentally, this can be confirmed by finding the effect of increasing the distance from muscle to soda. Normally the soda is put on a long strip of filter paper at a distance of about 3 mm. from the muscle. By putting the filter paper at the bottom of the bulb (with strict precautions to avoid getting any trace of soda on the sides of the vessel) the average diffusion distance can be increased about 10 times. The curve, nevertheless, remains unaltered: there is not even any discrepancy in the later stages when the carbon dioxide is appearing outside the muscle. No correction, therefore, is needed for carbon dioxide production.

*Correction of the record for time taken for diffusion of oxygen into the muscle.* The oxygen is used by the muscle cells at a rate greater than is apparent from the record. The muscle is assumed to use oxygen uniformly throughout its substance, and if its thickness and the diffusion constant of oxygen are known, a correction can be made. The method employed for correction is exactly similar to that used by Hill & Hartree [1920, etc.] for

analysis of galvanometer curves. The "control curve" in this case is calculated: it is that which would result from instantaneous consumption of a unit of oxygen by an infinite plane sheet of muscle [Hill, 1928, p. 68]. Krogh's value of the diffusion constant is used, corrected to  $0^{\circ}\text{C}$ . The thickness of the muscle is found by measuring the mass, and the length and breadth at several points while it is in position on the frame. The breadth is only 3-4 times the thickness, so that a slight error will result from use of the calculated "control curve", which is theoretically for an infinite plane sheet.

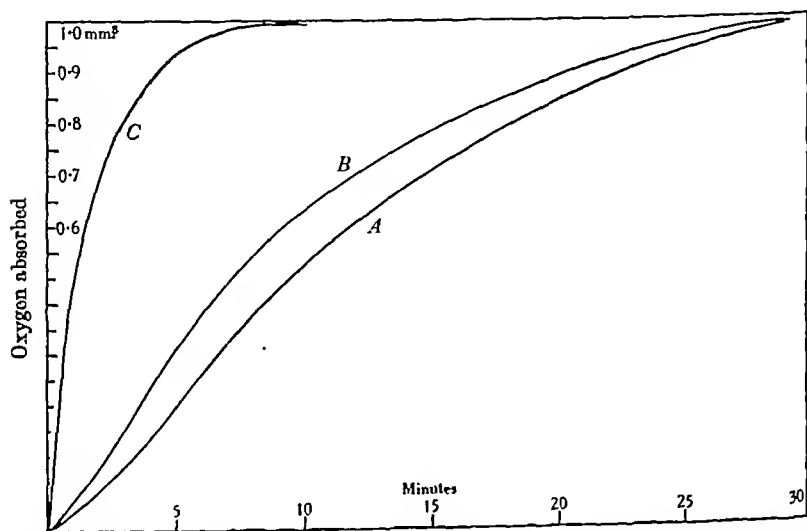


Fig. 5. *A*, the record uncorrected except for initial heat. *B*, after correction for diffusion. The muscle was of thickness 0.8 mm. *C*, the curve which would be followed by the index if consumption of oxygen coincided with the activity.

In Fig. 5 is shown the difference between the curves before and after analysis: the time to half maximum of the actual record is about 9 min. and of the corrected oxygen consumption curve is about 7 min. The oxygen uptake is complete in about 30 min. Included in Fig. 5 is the curve which would be followed by the index if oxygen utilization coincided with contraction.

*Comparison with recovery heat production.* Having obtained the time course of oxygen consumption of the muscle due to activity, it is of interest to compare this with the time course of oxidative delayed heat under precisely the same conditions. By oxidative delayed heat is meant that part of the delayed heat which is absent in the absence of oxygen: it is

derived by subtracting the anaerobic delayed heat from the total delayed heat (see a subsequent paper). Fig. 6 shows that the curves of oxygen consumption and oxidative heat production are closely similar. In the early stages there is no detectable difference between them: in the later stages there is a slight difference.

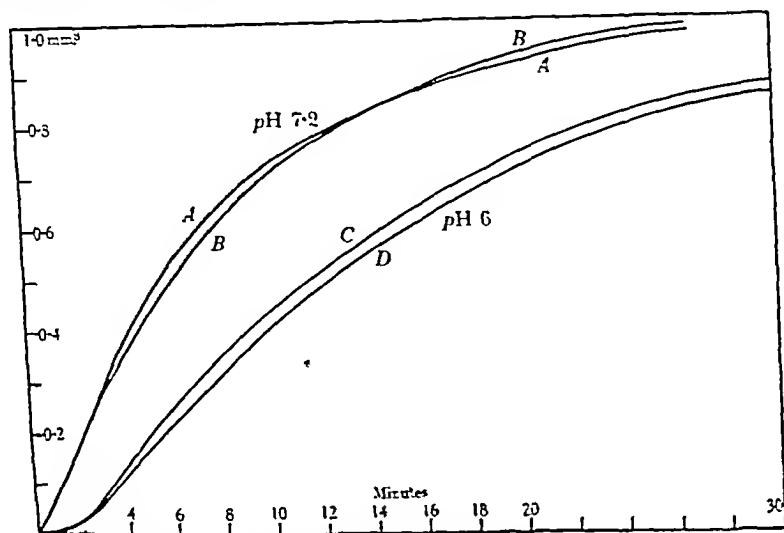


Fig. 6. Comparison of the time courses of oxygen consumption and of heat production. At pH 7.2: A, oxygen; B, heat. At pH 6.0: C, heat; D, oxygen.

#### *Modification of the time course of oxygen consumption following stimulation*

At a given temperature, modification of the time course can be brought about:

- (1) By change of hydrogen-ion concentration.
- (2) By change in duration of stimulus.
- (3) By the action of drugs.

The correction for diffusion is not large and for purposes of comparison of time courses of oxygen consumption under various conditions it need not be made. This applies to all subsequent parts of the paper.

*Recovery at high hydrogen-ion concentration.* It was shown by Hartree & Hill [1924b] that the effect of raising the hydrogen-ion concentration is to decrease the rate of delayed heat production. The determination of the time course of oxidative delayed heat production at 0° C. and pH 6 will be described in a subsequent paper. The time course of oxygen consumption has been determined under exactly the same conditions, namely, with the same duration of tetanus and with the same buffer mixture

containing 25 mg.P/100 c.c. The rates of oxygen consumption and of delayed heat production are much less than at normal pH, being about halved: also the time courses of the two processes are nearly identical (Fig. 6). At pH 6 no oxygen is used during the first minute following stimulation. This shows very clearly that the oxygen consumption is due entirely to a recovery process.

*The dependence of the time course of activity oxygen consumption upon duration of tetanus.* With a tetanus of duration less than 20 sec. the form of the oxygen consumption curve is invariable, and the rate of oxygen

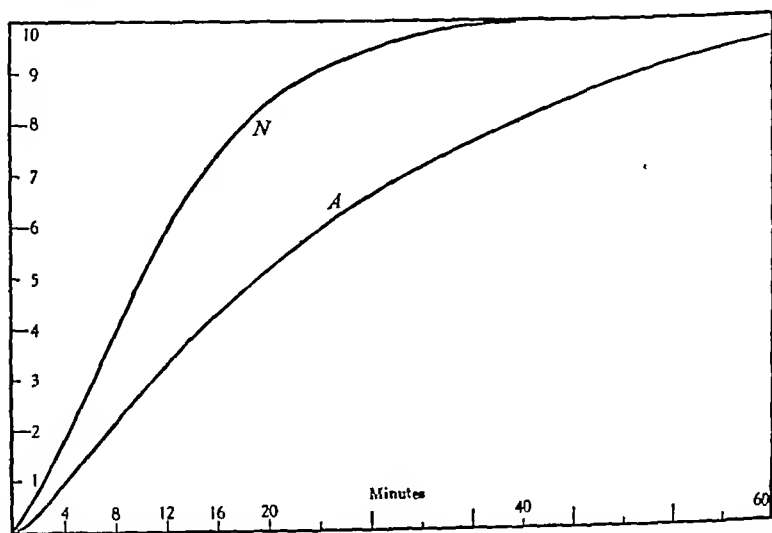


Fig. 7. The time course of oxygen consumption following a tetanus of duration 1 min. at pH 7.2. *N*, the same, but tetanus duration 12 sec. Curves scaled to same maximum.

consumption at any time is very closely proportional to the amount of recovery still to go, i.e. the curve has a logarithmic form. In the subsequent paper it will be shown that the same applies to the oxidative delayed heat at 0° C. These facts by themselves might point to the following simple theory. Activity causes the formation of a substrate *S* which combines with oxygen at a constant potential, and the rate of oxidation is equal to  $kS$ , where  $k$  is a constant. With this hypothesis there should be no limit to the rate of oxygen consumption: it should increase indefinitely with the duration of the stimulus. The failure of the simple theory is shown by the following facts:

(1) The form of the oxygen consumption curve following activity is not really independent of duration of tetanus. As the latter is increased

beyond about 20 sec. this fact becomes apparent: the rate relative to the amount of initial breakdown is found to diminish (Fig. 7).

(2) There is an upper limit to the rate of oxygen consumption induced by activity.

Both these facts are manifestations of the dependence of the substrate oxidation upon an enzyme system of limited capacity.

*Determination of the maximum possible rate of oxygen consumption.* The muscle is stimulated in pure oxygen for 0.2 min. every minute. After about 7 min. the rate of oxygen consumption reaches a constant level. The fact that recovery from a single tetanus takes about 30 min. suggests that with the greater amount of activity the maximum possible rate of oxygen consumption is reached. This is confirmed by three facts:

(1) By increasing the amount of stimulation the rate cannot be increased further.

(2) The rate of oxygen consumption during a 1 min. period, when corrected for heating, is uniform.

(3) When stimulation ends, the rate of oxygen consumption remains constant at its maximum level for about 10 min. It then declines and the oxygen consumption is not complete for another hour.

Time has not permitted the determination for many different muscles of the value of the maximum possible rate of oxygen consumption. It shows considerable variation from muscle to muscle. This is not incompatible with the very marked constancy of the time course of oxygen consumption following a short tetanus. Whereas the rate of oxygen consumption following a long tetanus depends chiefly upon the concentration of enzyme, this is not the case after a short tetanus, when the amount of substrate produced is small compared with the amount of enzyme present. For a typical experiment at pH 7.2 and 0° C. the maximum possible rate was found to be  $4.1 \times 10^{-3}$  c.c./g.  $\times$  min. The muscle was of thickness 0.86 mm. exposed on both sides, and the calculated maximum permissible steady rate of oxygen consumption for adequate supply is  $11.9 \times 10^{-3}$  c.c./g.  $\times$  min. The maximum possible rate of oxygen consumption is, therefore, not determined by the oxygen supply.

At pH 6 the maximum possible rate is much smaller than at pH 7.2, and is induced by less activity. A single 1 min. tetanus is enough. Following this the record is straight for about 10 min. (after correction for the initial heating) and the actual rate is found to be the same as that due to intermittent stimulation. In a typical experiment the value of the maximum rate was found to be  $1.4 \times 10^{-3}$  c.c./g.  $\times$  min., which is about one-third of that at pH 7.2. The variation of the maximum rate with pH confirms

the conclusion that the maximum rate is not limited by diffusion of oxygen.

*The effect of caffeine on recovery from activity.* Caffeine, in doses insufficient to produce contracture, causes an increase in the resting rate of oxygen consumption of isolated frog's muscle [Fenn, 1931; Saslow, 1937]. It was shown by Stannard [1939] that this *extra* oxidation is under the control of the enzyme system which is normally concerned only with the oxidation associated with activity. This conclusion can be tested by finding whether the oxidative recovery of a caffeinized muscle is abnormally slow.

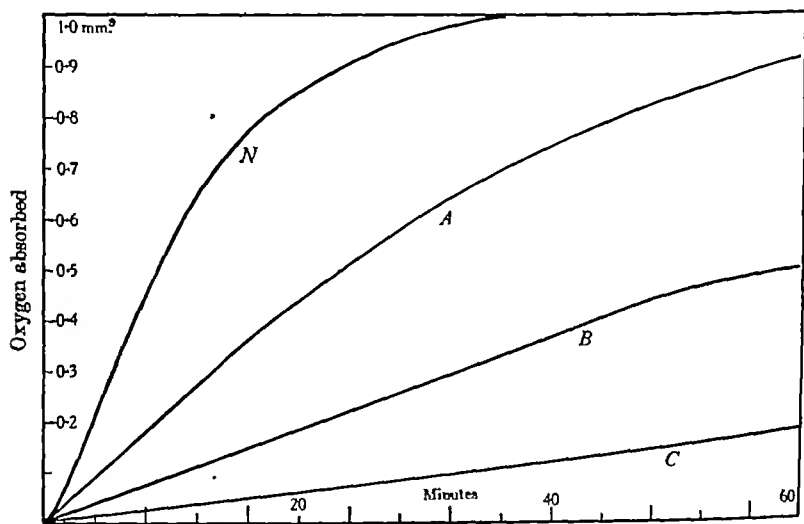


Fig. 8. The effect of caffeine on activity oxygen consumption. *N*, normal untreated. *A*, after treatment with 0.02% caffeine. *B*, 0.03% caffeine. *C*, 0.04% caffeine. Duration of tetanus in every case 0.2 min.

If the enzyme system responsible for oxidative recovery is being partially saturated by substrate supplied by the action of caffeine, then there must be less latitude for increase produced by stimulation. It is, therefore, of interest to examine the time course of the activity oxygen consumption of stimulated caffeinized muscle.

The muscles are soaked for at least 12 hr. in plain Ringer's solution. They are then immersed for 30 min. in the caffeinized Ringer's solution: this time should be long enough to allow diffusion of the caffeine to all parts of them. The results of 0.2 min. tetani with different concentrations are shown in Fig. 8. After treatment with a smaller concentration of caffeine (0.02%) the maximum rate of extra oxygen usage due to activity relative

to the total consumption due to activity is abnormally small. This maximum rate is maintained for about 15 min. and is, in fact, the maximum possible rate, as can be confirmed by demonstrating the impossibility of increase by further stimulation. With higher concentrations of caffeine the maximum rate of extra oxygen consumption due to activity becomes smaller. With 0.04% caffeine recovery from a 0.2 min. tetanus is so slow that it cannot be followed accurately. It was under such conditions that Hartree & Hill [1924a] and Saslow [1937] discovered an absence of delayed heat following stimulation. With concentrations greater than about 0.04% the muscle develops a contracture.

It would be interesting to ascertain whether, at any given caffeine concentration, the sum of (a) the maximum possible extra rate of oxygen consumption due to activity and (b) the resting rate, is constant and equal to the same quantity in a normal muscle. Accurate experiments, however, on these lines are impossible on account of the continuous decline in the value of the resting rate of oxygen uptake with a given concentration of caffeine.

#### *The respiratory enzyme system and recovery from activity*

*Sodium azide.* There are numerous reasons for believing that the activity respiration of muscle occurs with the participation of enzymes of the cytochrome complex. One of the chief of these reasons is that certain drugs, known to have an affinity for cytochrome oxidase, possess the power of inhibiting the activity oxygen usage of muscle. It is important to stress that this only applies to *activity* oxygen consumption, for Stannard [1939] has shown that resting oxygen consumption cannot be inhibited by sodium azide, although this substance is capable of putting cytochrome oxidase out of action and of inhibiting activity oxygen consumption. Stannard has investigated the action of concentrations of azide insufficient to cause total inhibition. When the concentration in the Ringer's solution is reduced to  $M/100,000$  he finds that there is no inhibition. With concentrations between this and  $M/1000$  the inhibition increases in such a way as to indicate the existence of a true stoichiometric equilibrium between the azide and the respiratory enzyme (presumably cytochrome oxidase), in which one molecule of the azide combines with one molecule of the enzyme.

It is of interest, therefore, to investigate the effect on the time course of activity oxygen consumption of concentrations of azide between  $M/100,000$  and  $M/1000$ .

The sartorii of a frog, previously soaked overnight in oxygenated Ringer's solution, were put into the azide-Ringer mixture for about 20 min.



before use. The oxygen consumption was then determined as usual after 0.1 min. tetanus. The muscles were then taken out and soaked in a solution of different strength. The process could be repeated a number of times using different concentrations of azide. Owing to the permanent presence of the resting oxygen consumption, the muscles remained in excellent condition, even with the higher concentrations of azide. In Fig. 9 are

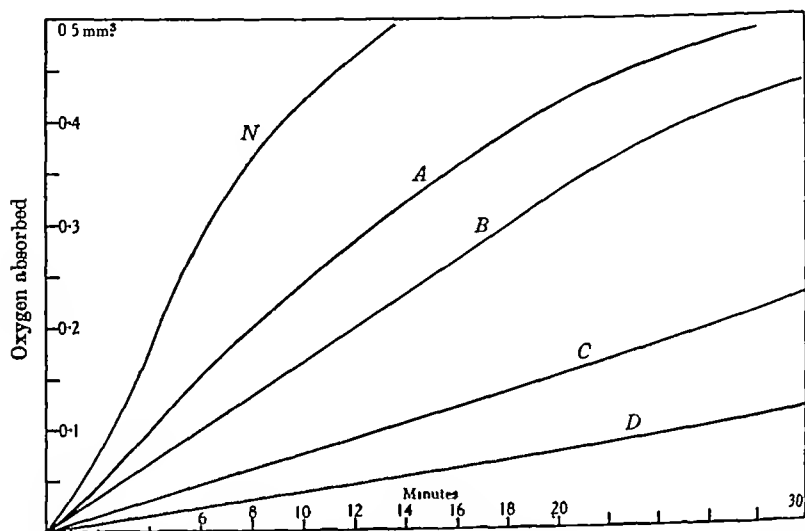


Fig. 9 The effect of sodium azide on activity oxygen consumption. *N*, normal untreated. *A*, after soaking in *M*/50,000 azide; *B*, *M*/25,000 azide, *C*, *M*/20,000 azide; *D*, *M*/15,000 azide. All taken with one muscle. At each concentration the duration of tetanus is 0.2 min. and the total oxygen used for recovery is the same. With concentrations of azide *M*/25,000 and higher the rate of oxygen consumption is constant (the maximum possible) for at least 20 min.

shown the curves for oxygen consumption with concentrations of azide varying from *M*/50,000 to *M*/15,000. A normal curve is included for comparison. With the higher concentrations of azide the recovery is not complete for a very long time; with *M*/15,000 not for 3 hr. The records were not usually observed after 30 min., and in order to proceed with the series at a different concentration the recovery was hastened by soaking the muscle at room temperature in pure Ringer's solution. It is important to be certain that complete recovery has occurred before commencing the next run: if this is not done, the activity oxygen usage will be apparently reduced.

With concentrations of azide higher than  $M/25,000$  the rate of oxygen consumption following activity is constant for a considerable period; with  $M/15,000$ , for example, for 30 min. When the rate is constant, it is the maximum possible, i.e. it cannot be increased by further stimulation.

The rate of oxidative recovery from activity is therefore a function of the effective concentration of cytochrome oxidase. The exact quantitative relation between azide concentration and maximum possible rate of oxygen consumption has not been explored. Stannard [1939] has investigated the relation between azide concentration and rate of oxygen usage by sub-contraction doses of caffeine, and there seems little doubt of the result. It might be better, however, to deal with the maximum possible rate of oxygen consumption rather than the rate produced by a given concentration of caffeine (or other drug); for only in the former case can one be certain that the cytochrome oxidase is being confronted with a fixed concentration of substrate—an assumption which is implicit in Stannard's argument. Otherwise confusion may result from the fact that the other enzymes taking part (e.g. the dehydrogenases or cozymase) are not working to capacity and the degree of saturation will be dependent upon the absolute rate of oxidation.

As mentioned, the relation between the maximum possible rate of oxygen consumption and azide concentration has not been investigated: the differential volumeter cannot be used effectively for the purpose. A constant rate of oxygen uptake is best determined with a muscle immersed in Ringer's solution, so that the composition of the intercellular fluid is constant. When azide is present, this is particularly important, because its power of inhibition is largely dependent upon  $pH$  [Keilin, 1936] being much greater in an acid than in an alkaline medium. The reason for this is that  $HN_3$  is probably the active agent [Armstrong & Fisher, 1939]. The dissociation constant of  $HN_3$  at  $0^\circ C.$  is  $10^{-5}$ , so that in the physiological range of  $pH$  the amount of  $HN_3$ , for a given azide concentration, will be nearly proportional to the hydrogen-ion concentration: the power of inhibition corresponds.

The changes in  $pH$  of an active muscle suspended in gas will be dealt with in a subsequent paper: they are due mainly to breakdown and re-synthesis of phosphocreatine and to lactic acid formation. Not only will the degree of inhibition of cytochrome oxidase vary during the cycle of recovery, but the time course of the variation will depend upon the azide concentration and the duration of stimulus. In the subsequent paper it will be shown that, with a given azide concentration, the duration of stimulus is the factor determining whether or not lactic acid is formed.

This is illustrated by comparing the rates of oxygen consumption following a 0.5 min. tetanus of a muscle treated with  $M/5000$  azide. The smaller duration of stimulus is sufficient to induce the maximum possible rate of oxygen consumption: the only effect of the longer tetanus is to cause the appearance of lactic acid. This augments the inhibition of the oxidase and increases the rate of production of lactic acid and so on. This vicious circle is manifested by a very rapid reduction in rate of oxygen consumption following a long tetanus with azide concentration about  $M/5000$ . It will be shown that unless the duration of tetanus exceeds a critical value (which depends upon the azide concentration) formation of lactic acid is *entirely* absent. For the runs shown in Fig. 9 the critical value was not exceeded, so that the only  $pH$  changes were due to phosphocreatine breakdown and were therefore to the alkaline side: this does not produce the vicious circle referred to.

By this method, therefore, quantitative results cannot be procured. The only accurate way of dealing with the problem is one with the muscles in buffered Ringer's solution.

Apart from the above-mentioned disadvantage connected with the use of azide, there is much to commend its employment as an inhibitor of cytochrome oxidase activity. It is soluble, non-volatile, diffusible and the muscle can quickly be freed from it by washing. Its properties, in relation to cytochrome oxidase, are well established [Keilin, 1936; Keilin & Hartree, 1939]. It might be thought possible to avoid the complication with  $pH$  changes by using cyanide as the respiratory poison. Although it is probable that undissociated  $HCN$  is the active agent, the dissociation constant is small enough ( $7.2 \times 10^{-10}$ ) for there to be no appreciable alteration of inhibition due to  $pH$  changes in the muscle. It is the volatility of  $HCN$  which renders the use of cyanide impossible, for it distils from the muscle to the soda on the wall. To prevent such distillation it would be necessary to mix cyanide with the soda so that the  $HCN$  concentration was the same as in the muscle. It can be calculated that this would require the addition of  $10N$  cyanide to the soda: this is impracticable because the muscle would rapidly lose water by distillation to the hypertonic solution.

*Sodium malonate.* Enzymes other than those of the cytochrome complex take part in the oxidative reaction chain in muscle: such are the coferments triphosphopyridine nucleotide and diphosphopyridine nucleotide, which are responsible for transferring electrons from hexose phosphate to the "yellow ferment" and to cytochrome *c*. Little is known about other systems, but there is evidence of their existence: for example, pyruvic and

lactic acids can be oxidized by muscle, and the utilization of fuel other than carbohydrate is indicated by a respiratory quotient less than unity [Gemmill, 1934]. Specific inhibitors of the enzymes for the reactions mentioned are not available, and the dependence of the rate of oxidative recovery upon these systems cannot be gauged. The action, however, of sodium malonate has been investigated: this substance is an inhibitor of succinic dehydrogenase, an enzyme noted for its ubiquity, but not known to have any function in muscle. The concentration of sodium malonate required to produce a reduction in rate of oxygen consumption following activity is high.  $M/500$  malonate in the Ringer's solution causes only a slight inhibition: as the concentration is raised to  $M/50$  the inhibition increases and oxidative recovery from activity is delayed. The effect becomes more marked with successive runs at one concentration without intermediate soaking, until activity oxygen consumption may be almost abolished.

The results are interesting, but their significance is doubtful.

#### DISCUSSION

The demonstration of the identity of the time courses of oxidative delayed heat production and oxygen consumption carries more significance than is immediately obvious. The delayed heat is the sum of the heats from two processes, namely the oxidative catabolism (exothermic) and the endothermic resynthesis. (The latter is the reversal of the reactions which are the source of the initial heat.) It follows that these two processes must individually follow the same time course as the oxygen consumption (there is the unlikely possibility that the divergence of one is compensated by an opposite divergence of the other). In fact there must be rigid coupling between the process of resynthesis of phosphocreatine and the oxidative reaction which drives it. It is well known that the resynthesis of phosphocreatine occurs as a reaction coupled with oxidation of triose-phosphate to phosphoglyceric acid (with triphosphopyridine nucleotide as coferment) and with the further disintegration of the latter substance, yielding pyruvic acid which is also oxidized. The inference is that activity oxygen consumption occurs mainly through these channels. The maximum possible rate of oxygen consumption may well be an index of the concentration of triphosphopyridine nucleotide, but so many enzyme systems are involved that such speculation is to be avoided.

An alternative means of oxidation of triosephosphate to phosphoglyceric acid is afforded by reduction of pyruvic acid to lactic acid with diphosphopyridine nucleotide as coferment. This alternative route in

relation to recovery from activity will be considered in a subsequent paper.

The experiments with azide strongly suggest that the rate of oxidative recovery from activity is dependent upon the concentration of cytochrome oxidase. It would be of interest to examine the effect of raising the concentration of cytochrome *c* in the muscle. This member of the cytochrome complex can be obtained in a pure state and its molecular weight is such as to permit its entry into the cell. It can be oxidized only via cytochrome oxidase [Keilin & Hartree, 1938]. Methods could also be devised for introduction of an oxidation-reduction system of the ferri-ferro type such as methaemoglobin-haemoglobin. This is autoxidizable and of a potential approximately equal to that of cytochrome *c* and might function as a substitute. In this way it might be possible to obtain an increase in the rate of oxidative recovery.

The capability of an animal for prolonged heavy effort must be related to the rate of oxidative recovery of its muscles and heart. Such capability shows great variation from phylum to phylum and species to species and is paralleled in the muscular content of cytochrome. The wing muscles of an insect are very rich in cytochrome and those of a frog comparatively poor. Preliminary experiments have been done on the isolated wing muscles of the locust to determine the rate of oxidative recovery from activity: results obtained just before the present experiments had to be broken off owing to the outbreak of war show that this rate is very much greater than with a frog's muscle at the same temperature. An idea of the relative rates of metabolism is given by the fact that the resting rate of oxygen usage by the locust muscle is about equal to the maximum possible rate of oxygen usage of a stimulated frog's muscle. In fact the difference is probably of the order of a hundredfold! There is here a wide field for research.

As regards athletic ability and cytochrome content, the mammal comes between the frog and the locust (at 37° C.). With this fact in mind the results given in this paper may perhaps be used as a basis for estimating the difficulty of the parallel problem in warm-blooded muscle.

#### SUMMARY

1. A method of determining the time course of the extra oxygen consumption of stimulated frog's muscle is described. There was always an excess of dissolved oxygen available in the muscle.

2. To permit a resolution of phases the metabolic processes are slowed by working at 0° C.

3. The oxygen consumption occurs entirely after activity. With a tetanus of less than about 20 sec. it is half complete in 6 min. and complete in about 30 min.

4. The time course of oxygen consumption is the same as that of oxidative delayed heat under the same conditions. The significance of this is discussed.

5. The time course of oxygen consumption depends upon the duration of stimulus. The greater the duration the more delayed is the oxidative recovery. This fact only becomes apparent if the duration of tetanus exceeds about 20 sec.

6. As a corollary to 5, there is found to be a maximum possible rate of oxygen consumption which depends upon pH but is not determined by the rate of diffusion of oxygen.

7. Consistent with this is the action of caffeine on the time course of activity oxygen consumption.

8. By means of sodium azide it is possible to inhibit the action of cytochrome oxidase to any extent desired and correspondingly to decrease the rate of recovery from activity.

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## DETERMINATION OF THE METABOLIC RATE OF ALCOHOL

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The rate at which alcohol is oxidized in the body is measured approximately by the rate at which it disappears, since the proportion excreted is small and regular; and the rate at which it disappears is equal to the body weight multiplied by the rate of change of concentration in the whole body. In man, however, the rate of change of concentration can be measured only in the blood. Hence, it is of importance to know the relation between the concentration in the blood and the concentration in the whole body at the same moment. If this relationship is not reasonably constant, the extent and time relations of any variation must also be known.

The proportion of the body in alcohol equilibrium with the blood (the mean concentration in the whole body expressed as a fraction of blood concentration) has been designated  $\tau$  by Widmark [1932] and derived from the change in blood alcohol concentration in the following manner (see Fig. 2). The straight line joining the experimental points is extrapolated back to zero time, giving the theoretical initial concentration in the blood ( $C_0$ ): the amount of alcohol injected and the weight of the subject are measured; then

$$\tau = \frac{\text{alcohol injected (g.)}}{\text{body weight (kg.)} \times C_0 \text{ (mg./g.)}}.$$

The metabolic rate of alcohol is, therefore,  $\tau$  multiplied by the rate of increase in blood alcohol concentration (designated  $\beta$  by Widmark). This value  $\tau$ , and the metabolic rate calculated from it, are intimately dependent on the line extrapolated from the blood alcohol curve and therefore on the regularity of the experimental points from which that

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curve is constructed. Moreover, the assumption is implicit that the blood is in equilibrium with the same proportion of the body tissues throughout the experiment, and it might be expected that the same individual should show the same value of  $r$  during different experiments performed within a short interval of time. In most of the work published, in which Widmark's [1922] micro-method of alcohol estimation is used, these conditions are not fulfilled. Irregularity in the experimental points of the blood alcohol curve renders extrapolation of any straight line drawn through them a dubious matter, and, possibly because of this, the value of  $r$  for any individual varies within quite wide limits, e.g. 0.52–0.67 in man [Bernhard & Goldberg, 1935] and 0.7–0.8 in the dog [Widmark, 1933].

Some factors, other than analytical, affecting the regularity of the decrease in blood alcohol concentration have been suggested by various workers. It seems likely that analysis of venous blood gives less regular results than that of arterial blood, presumably because the circulation in some tissues is less effective than in others, and since these tissues (e.g. muscle) are not using alcohol, blood which has lingered there will contain a higher concentration of alcohol than blood in the general circulation which has passed through the liver. The same explanation, extended possibly to the spleen, covers the observations of Nyman & Palmłow [1934], who found that in subjects who had been completely resting, renewed activity actually produced a slight increase in the concentration of alcohol in the blood instead of the expected decrease with the passage of time. In addition, the decrease in the concentration of alcohol in the blood is a result not only of its metabolism but also of its excretion. In the present series of experiments, excretion in the urine accounted as a rule for  $1\frac{1}{2}$ –2 % of the alcohol injected, occasionally rising as high as  $3\frac{1}{2}$  % and no correction in the value of  $\beta$  was considered necessary.

In view of the importance of this factor  $r$  in the measurement of the metabolic rate of alcohol, experiments were designed to test the validity of its derivation from the change in blood alcohol concentration, and its constancy in any one individual.

## METHODS

Cats were used as experimental animals, and were given only water for the 18 hr. previous to experiment. They were anaesthetized with nembutal, given intraperitoneally (0.65 c.c./kg.), and thereafter maintained at constant body temperature  $\pm 0.5^\circ\text{C}$ . Alcohol was usually injected intravenously in 5, 10 or 20 % solution in saline into the jugular



vein at a rate varying from 40–200 mg./min. Blood samples were removed from the carotid artery. The blood, collected in tightly stoppered tubes with oxalate, was placed immediately at 0° C., centrifuged within a few hours, and the plasma returned to the refrigerator until analysis the following day. If kept in tightly stoppered small vessels, no appreciable loss of alcohol occurred during several days at 0° C. Muscles were removed after ligature, ground immediately (after being weighed) with a measured volume of cold 10 % trichloroacetic acid, and the mixture left at 0° C. in a stoppered centrifuge tube until centrifuged before analysis. In some cases they were first frozen in liquid air on removal from the body, a procedure always followed in the case of liver, which was removed at the end of an experiment.

Analysis of the alcohol content was then made on 1 c.c. plasma or on 1 c.c. acid filtrate of the tissues, in the following manner. The method used was based on that described by Newman [1936], but altered in certain details. In essence, the alcohol is distilled under reduced pressure from the plasma mixed with anhydrous  $\text{Na}_2\text{SO}_4$  into a standard  $\text{K}_2\text{Cr}_2\text{O}_7\text{-H}_2\text{SO}_4$  mixture, the excess of  $\text{K}_2\text{Cr}_2\text{O}_7$  being titrated with thiosulphate. In practice, standard conditions are found essential at many points for accurate recovery. (1) An excess of anhydrous  $\text{Na}_2\text{SO}_4$  is used to prevent frothing (a crude preparation should be used, since the A.R. material supplied by British Drug Houses Ltd. is not sufficiently finely dispersed for the purpose), and the plasma is added to it only shortly before analysis. (2) The tube connecting the distillation flask with the receiving tube is both dry and tilted downwards towards the receiving vessel. (3) The distillation flask is maintained at 50–55° C. at a pressure of 40–60 mm. Hg for 10–15 min., and the connecting tube then gently warmed before the vacuum is broken. An ordinary reading lamp on an adjustable stand suffices for this purpose. With a battery of six sets of apparatus connected with the same vacuum pump, the whole six estimations could be completed and the apparatus reassembled in 1¼–1½ hr. No special apparatus is required beyond 25 c.c. Pyrex Erlenmeyer flasks and 15–20 c.c. Pyrex collecting tubes, together with suitable two-way taps on each for adjusting the pressure. Rubber corks are used and the connecting tube is joined in the middle with ordinary rubber tubing. The receiving flask contains 5 c.c.  $N/20 \text{ K}_2\text{Cr}_2\text{O}_7$  in 50 %  $\text{H}_2\text{SO}_4$  and is titrated against  $N/40$  thiosulphate of which 1 c.c. is equivalent to 0.287 mg. alcohol. The technique described is adequate for determination of 0–280 mg. alcohol/100 c.c. plasma. The recovery of alcohol, from watery solution or plasma, is complete, and the discrepancy between

duplicate determinations is rarely greater than 2 mg./100 c.c. The presence of trichloroacetic acid in the distilling flask does not affect the estimation.

### RESULTS

#### *The relation between alcohol concentration in the tissues and in blood plasma*

Following an intravenous injection of alcohol into the body, equilibrium between muscles and blood is reached only after about 30 min. (Fig. 1). The time course of the alcohol concentration in the blood suggests, however, that equilibrium in the whole body is not complete for

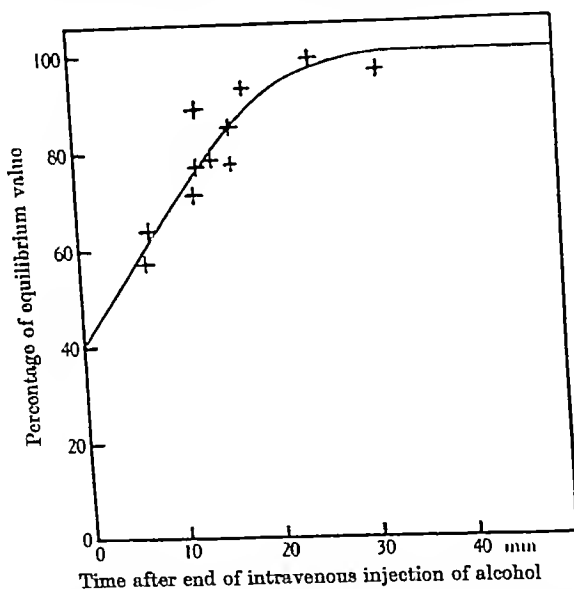


Fig. 1. The rate of equilibration between skeletal muscle and blood plasma following an intravenous injection of alcohol, as determined by direct analyses of the two tissues. The 100 % value is taken as the average equilibrium value obtained 1-6 hr. after an injection.

over an hour (Fig. 2); this difference may well be due in part to the delay which is known to occur in the establishment of equilibrium between the blood and cerebro-spinal fluid [Abramson & Linde, 1930]. When equilibrium has been reached, all the tissues analysed, with the exception of fat, show a concentration of alcohol 70-80 % of that in the blood plasma (Table I). If liquid air was not used in killing the tissue, a considerably lower value (55-65 %) was obtained with liver, as recorded in previous literature. No such difference was observed in the case of muscle. This

is to be expected in view of the discovery that at least 90 % of the metabolism of alcohol occurs in the liver and none in the muscles [Lundsgaard, 1937].

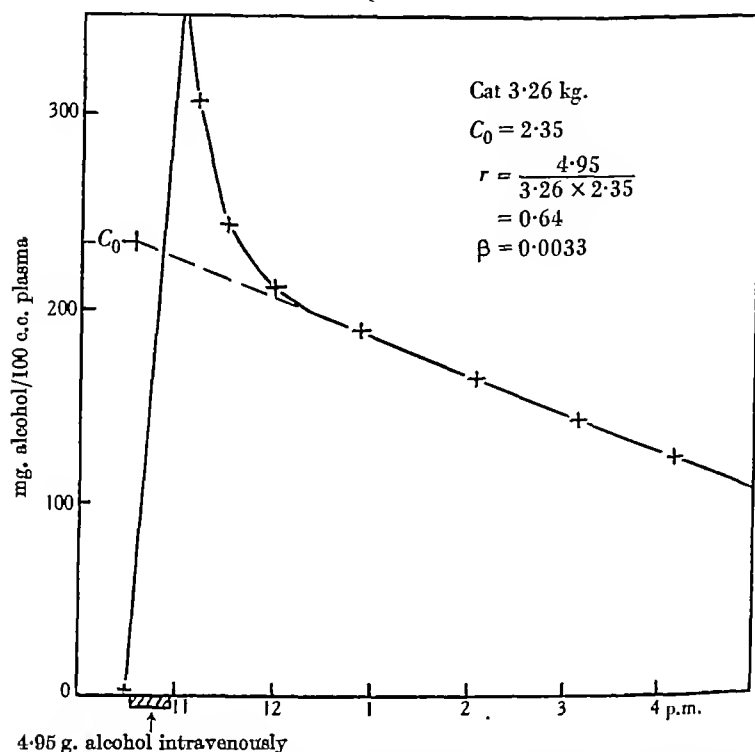


Fig. 2. The alcohol concentration in blood plasma following an intravenous injection of alcohol, showing Widmark's method of determining the factor  $r$  and thus the metabolic rate. Equilibrium between tissues and blood is not complete until ca.  $1\frac{1}{2}$  hr. after the end of the injection.

TABLE I. The relation of the concentration of alcohol in tissues to that in plasma after equilibrium is established.

Tissue	Ratio of concentration of alcohol in tissue to that in plasma (per g. of tissue)	Standard error	No. of observations
Skeletal muscle	0.74	$\pm 0.007$	26
Liver	0.73	$\pm 0.02$	5
Kidney	0.77	$\pm 0.01$	4
Intestine	0.755	$\pm 0.01$	6
Spleen	0.8, 0.81	—	2
Brain	0.785	$\pm 0.01$	4
Fat	0.16	$\pm 0.03$	8

The low value for fat, corroborating previous workers, was unchanged whether the alcohol was estimated directly by distilling the fat under reduced pressure, or by previous extraction with water or trichloro-acetic acid and distillation of the watery extract. It is stated in the literature [Smith & Stewart, 1932] that bone also contains little alcohol as compared with the blood, and the varying proportions of bone and fat in different individuals would readily explain the widely differing values of  $r$  observed by the indirect method.

With a view to determining the constancy of  $r$  during any one experiment, muscles were removed at varying times. The ratio of the concentration of alcohol in muscle and plasma remained appreciably unchanged for 6 hr. or so after equilibrium had been reached, and was also unaffected by the absolute concentration of alcohol in the body (Table II). A similar constancy of the ratio of the concentration of

TABLE II. The relation of the concentration of alcohol in muscle to that in plasma, (A) at varying times after the injection, and (B) at varying concentrations of alcohol.

A	
Time after alcohol injection hr.	Ratio of concentration of alcohol in muscle to that in plasma (per g. of tissue)
0.5-1	0.715 $\pm$ 0.02 (6)
1-2	0.70 $\pm$ 0.02 (5)
2-3	0.725 $\pm$ 0.02 (5)
3-4	0.74 $\pm$ 0.02 (8)
4-5	0.76 (1)
5-6	0.76 (1)
6-7	0.75 $\pm$ 0.02 (8)
B	
Alcohol concentration mg./100 c.c.	Ratio of concentration of alcohol in muscle to that in plasma (per g. of tissue)
100-200	0.75 $\pm$ 0.02 (10)
200-300	0.725 $\pm$ 0.01 (16)
300	0.715 $\pm$ 0.02 (8)

alcohol in both brain and liver tissue to that in the blood during  $\frac{1}{4}$ -12 hr. following a dose of alcohol was observed by Harger, Hulpieu & Lamb [1937] in dogs.

*Assessment of the metabolic rate of alcohol from  
changes in the plasma alcohol concentration*

From these direct analyses, one would expect the values obtained by indirect assessment of  $r$  to lie between 0.2 and 0.8, lean muscular individuals showing a higher value than obese ones. This is in fact the

case. Average figures obtained by Widmark [1933] were 0.68 for men and 0.55 for women. In the present series of cats,  $r$  varied from 0.48 (a very large, fat cat) to 0.78 (a young, muscular one) with an average value of  $0.59 \pm 0.02^1$ . These values are not strictly comparable with those of previous workers since the terms  $\beta$  and  $r$  are used in a slightly different manner from that introduced by Widmark.  $\beta$ , the decrease in alcohol

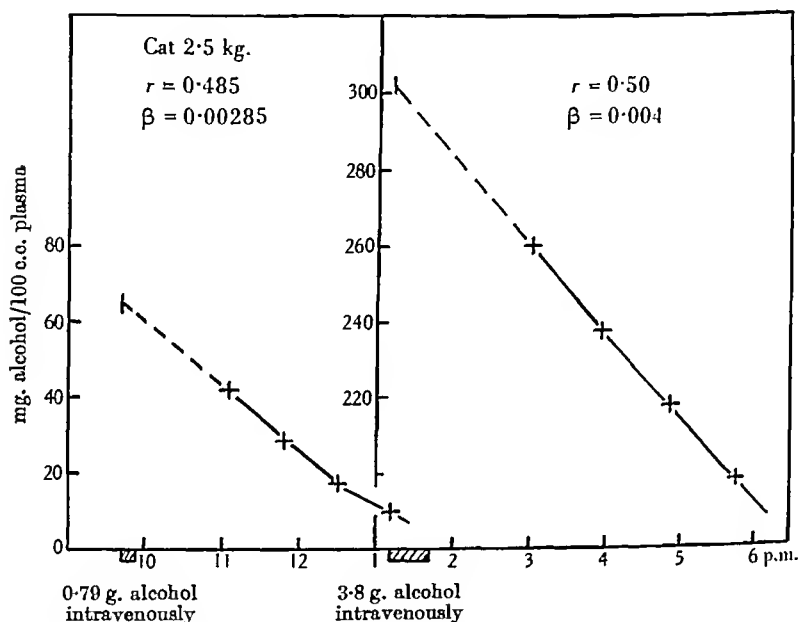


Fig. 3. The concentration of alcohol in blood plasma following two separate intravenous injections of alcohol, showing the identity of  $r$  in the two cases.

concentration in the blood, is expressed as mg./c.c. plasma/min. instead of mg./g. blood/min., and  $r$  is the proportion of the body in equilibrium with the blood plasma instead of with the whole blood. Alcohol concentration is 10–20 % higher in the plasma than in whole blood (in man; [Miles, 1922]) and the value of  $r$  as used in this series of animals is, therefore, possibly 10–20 % lower than that of Widmark.

If the large variations of  $r$  in any one individual noted by other workers are real, the question of the time factor of this variation becomes of importance, for obviously if  $r$  varies from hour to hour the whole method of assessment of metabolic rate from changing concentration of alcohol in the blood is suspect. In twenty animals (nineteen cats, one

<sup>1</sup> s.e. of mean is given here and elsewhere in the paper.

dog)  $r$  was determined twice, a second alcohol injection being given after the first value of  $r$  had been obtained, as shown in Fig. 3. In no pair of values on the same animal was a difference greater than 0.035 observed. The second determination was higher than the first in nine animals, lower in seven, and the same in four: the mean difference between the second and the first value in each of the animals being  $+0.003 \pm 0.004$ . The standard deviation from the mean of the difference between any pair of determinations is  $\pm 0.018$ , and it seems unlikely that the errors involved in the whole experimental technique would be less than this.

*Assessment of the metabolic rate of alcohol by constant infusion*

The reliability of the assessment of the metabolic rate of alcohol from the observed value of  $\beta$  and inferred value of  $r$  was subjected to a further test. The procedure shown in Fig. 2 was adopted in the first half of the experiment, so that the metabolic rate could be calculated in the usual way; a constant intravenous infusion of alcohol was then given over a period of hours, at a rate calculated to equal approximately the metabolic rate. The infusion apparatus used was a 20 c.c. syringe operated by a screw, driven by a geared motor. In the first experiment, in which 1.9 g. alcohol/kg. was injected into a 2.62 kg. cat,  $r$  was 0.655 and  $\beta$  0.0047 in the hour preceding the constant infusion, at which time the plasma alcohol concentration was 149 mg./100 c.c. The calculated metabolic rate was thus 186 mg./kg./hr. The infusion which followed (5.5 c.c./hr. of 8.5 % alcohol in saline) was 179 mg./kg./hr. After 3 hr., the plasma alcohol concentration had risen by only 2 mg./100 c.c. This difference is within the limits of experimental error of the method, but supposing it to be real and the additional 2 mg./100 c.c. distributed throughout the body, then 20 mg./kg. of the alcohol infused remained unmetabolized during 3 hr., or 7 mg./kg./hr. The metabolic rate, therefore, lay between 172 and 179 mg./kg./hr., and the metabolic rate assessed from the decrease in plasma alcohol concentration was 4–8 % higher than the true value.

In a second experiment of the same nature, the plasma alcohol concentration rose from 130–132 mg./100 c.c. over a period of  $3\frac{1}{4}$  hr., indicating the accumulation of about 6 mg./kg./hr. During this period, 154 mg./kg./hr. had been infused, and the metabolic rate lay, therefore, between 148 and 154 mg./kg./hr. Values of 0.59 for  $r$  and 0.004 for  $\beta$  obtained earlier in the experiment yielded a value of 141 mg./kg./hr. In this case, therefore, the assessed metabolic rate was 4–8 % lower than the true value. In view of the errors, both physiological and chemical,

involved in such experiments as these, the results may be accepted as evidence that the value obtained by indirect assessment of metabolic rate is a fair approximation to the true value.

### DISCUSSION

The average ratio of the concentration of alcohol in the tissues examined (other than fat) to that in the plasma is about 0.75, and the average value of  $r$ , assessed from changes in plasma alcohol concentration, 0.59. It has been assumed that this latter value is what one might expect for the whole body, since the concentration of alcohol at any time is considerably less in bone and fat than in the other tissues; but no attempt has so far been made to correlate the two values quantitatively. This cannot be done with any accuracy, but an approximate calculation made from the data in Table I and the average proportion of the different tissues in the human body [Vierordt, 1893] yields a value of 0.6. This figure is also of the same order as that obtained by Widmark [1932] for men, 0.55–0.6 (when due allowance is made for the difference in concentration of alcohol in plasma and whole blood), from changes in the concentration of blood alcohol.

Observed values lower than 0.6 are readily understandable, for the body weight can be increased very considerably by the simple addition of fat, but higher values, as e.g. the maximum observed of 0.78, are more difficult to interpret. This particular value was obtained (by two separate determinations) in a 2 kg. kitten, and the two other values encountered above 0.7 (0.72 and 0.74) occurred also in young lean cats. Two possible factors may be concerned in the production of these high values. In the first place, the proportionate weight of the different tissues varies with age, and on the balance, tissues which contain a higher concentration of alcohol may predominate in the young. In the second place, the cats used (13) for the tissue analyses in Table I were larger than the average, and since fat is deposited to a greater or less extent in all tissues, the ratios obtained may be lower than would have been the case if young lean cats had been used. On the whole, the evidence available suggests that the values obtained by indirect assessment of  $r$  are compatible with the directly determined concentrations of alcohol in the tissues.

Although the evidence presented here suggests that  $r$  is a constant in any individual (apart from gross variations in body weight), a study has not been made of possible long term variations. It is difficult to imagine that the proportions of different tissues change materially in an animal of constant weight, or that variations in the value of  $r$  could

occur in the absence of such changes. Yet Widmark [1932] has noted that the raised metabolism of alcohol present in fever is due entirely to an increase in the value of  $r$ . He gave no details of possible changes in body weight, but the increase in  $r$  was too large (0.12 in one subject) to be readily accounted for by loss of body fat in the course of a few days. In the present state of knowledge, therefore, the possibility of relatively large changes in  $r$  occurring in normal healthy individuals and caused by some factor or factors unknown, cannot be dismissed.

### SUMMARY

1. A method is described for the estimation of alcohol in 1 c.c. of plasma or trichloroacetic acid filtrate of tissue, yielding full recovery, with an error of  $\pm 1$  mg./100 c.c.

2. Following an intravenous injection of alcohol into cats under nembutal anaesthesia, direct determinations on muscle and plasma indicate that equilibrium between them is established only after 30 min. From the shape of the blood alcohol-time curve, it would appear that complete equilibrium throughout the body is established only after  $1-1\frac{1}{2}$  hr.

3. When equilibrium is established, most tissues in the body contain 70-80 % of the concentration of alcohol present in the plasma. Fat contains only 10-20 %.

4. This equilibrium value between muscle and plasma is unaffected by the absolute concentration of alcohol, or by the passage of time (6 hr. or so).

5. If the alcohol concentration in the whole body, expressed as a fraction of that in the plasma (which is a modification of Widmark's factor  $r$ ) is estimated indirectly it is found to remain constant in any one animal, though it varies widely from one animal to another (0.48-0.78 in the present series), presumably owing to the widely varying proportion of fat in the body.

6. Assessment of the metabolic rate of alcohol from the modified factor  $r$  and the observed decrease in the concentration of alcohol in the plasma agrees, within the limits of experimental error, with the direct measurement of metabolic rate (constant infusion method).



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SOME FACTORS AFFECTING THE METABOLIC RATE  
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MOST of the older work on the metabolism of alcohol was concerned primarily with its concentration in the blood and the factors affecting the rate of change in this concentration. With Widmark's introduction of a method for assessing the actual metabolic rate of alcohol from its rate of change of concentration in the blood, greater attention has been paid to the more fundamental question of the factors affecting the metabolic rate. With rare exceptions, however, the results obtained by different workers are conflicting: there is as yet no unequivocal answer to the apparently simple questions: Is the metabolic rate of alcohol affected by its concentration in the body? Is it increased in the "tolerant" subject? Or by exercise? Or by the taking of food? One major difficulty in experiments designed to answer such questions is the fact that different individuals vary so greatly amongst themselves in the rate at which they metabolize alcohol. Either large numbers of control and experimental animals have to be used, therefore, and the results treated statistically, or the same animal has to be used for both control and experimental purposes.

The possibility of variations in the distribution of hormones accounting for some of the differences observed in the metabolic rate of alcohol has been considered by various workers [Widmark, 1935; Lang & Schlick, 1936], but no obvious connexion has been found. Men are known to metabolize alcohol faster than women on the average [Widmark, 1932], and damage to the liver is associated with a reduction in the metabolic rate (see discussion later). Apart from these observations, however, no

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systematic work has been done in attempting to assess the factors responsible for the large variation in the metabolic rate of alcohol encountered in different individuals. The results presented below represent an attempt to determine the effect on the metabolism of alcohol (1) the concentration of alcohol, (2) the size of liver, (3) previous feeding with alcohol, and (4) the amino acid, alanine.

### METHODS

The present work has been done mainly on cats, under nembutal anaesthesia, and the general physiological and chemical techniques have been already described [Eggleton, 1940].

### RESULTS

#### *Concentration of alcohol*

In spite of the reiteration by many workers that the decrease in concentration of alcohol in the blood with time is linear, and the metabolic rate of alcohol therefore independent of its concentration, there are several hints in the literature that this is not strictly true. Hagberg & Greenberg [1934], working with dogs, concluded that the metabolic rate is directly proportional to the concentration. Their technique is open to doubt, however, for calculation of  $r$  (by Widmark's method) gave values of 0.93 and 1.1 in two of their four experiments. This latter value indicates a concentration of alcohol in the whole body higher than in the blood, and is contrary to the results of all other workers. Newman & Lehman & Cutting [1937] gave doses of alcohol to dogs, covering a wide range of concentration in the blood, and found a more rapid decrease at the higher levels than at the lower. In spite of this they maintained that the rate of decrease was linear in every case, and were at a loss to reconcile the two sets of observations. Again, Elbel [1937] quotes two different workers from his laboratory finding a greater rate of metabolism in human subjects after larger doses of alcohol. Most workers on human subjects, however, are content with the linear hypothesis because their work is concerned with such a small range of concentration of alcohol in the blood, and frequently also because the scatter of the experimental points about the straight line is such that a small change in slope would pass unnoticed.

In the present series of experiments, in which alcohol concentrations in the plasma of 10-400 mg./100 c.c. were covered, there is

unmistakeable relationship between alcohol concentration and metabolic rate. The experiments on which this statement is based were of three kinds.

*Comparison of the metabolic rate at two widely varying concentrations induced by two separate injections of alcohol.* This type of experiment is illustrated in Fig. 1 and was performed on fifteen animals (one dog, nine normal and five "tolerant" cats). The magnitude of the change in metabolic rate with increasing alcohol concentration in the plasma can be

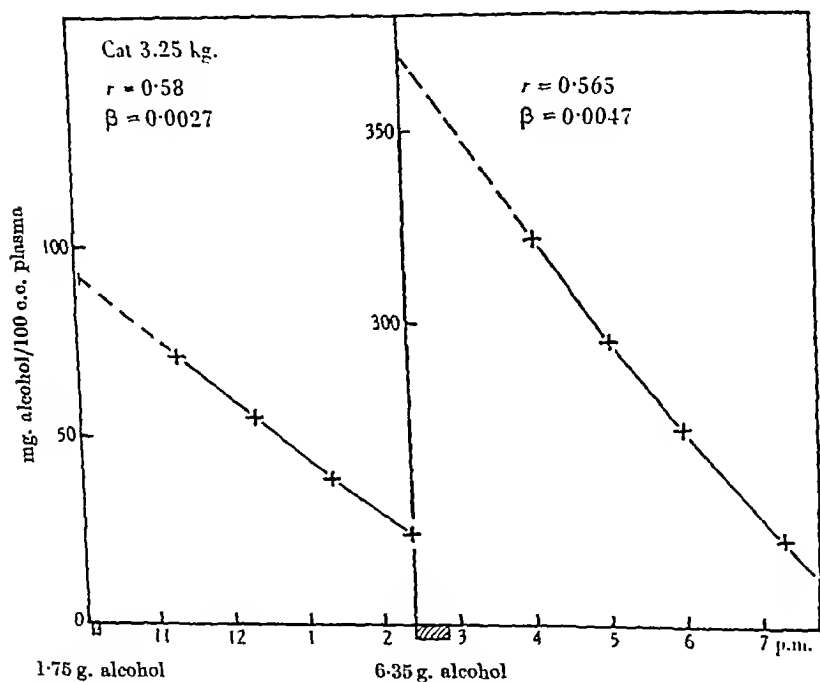


Fig. 1. The concentration of alcohol in blood plasma following two separate intravenous injections of alcohol, showing the faster rate of metabolism at the higher concentration.

seen in Fig. 2, in which the results of all the experiments are shown. In several experiments, intermediate values between the two extremes recorded in the figure were obtained. The same relationship between concentration and metabolic rate was seen in all, but they have been omitted from the figure for the sake of clarity. Above 20 mg./100 c.c., an increase of 100 mg./100 c.c. in plasma alcohol concentration was accompanied on the average by about a 30 % increase in metabolic rate. In

two experiments in which concentrations below 20 mg./100 c.c. were studied (values not recorded in Fig. 2), the change in metabolic rate was considerably greater than this, a result confirmed in other types of experiment.

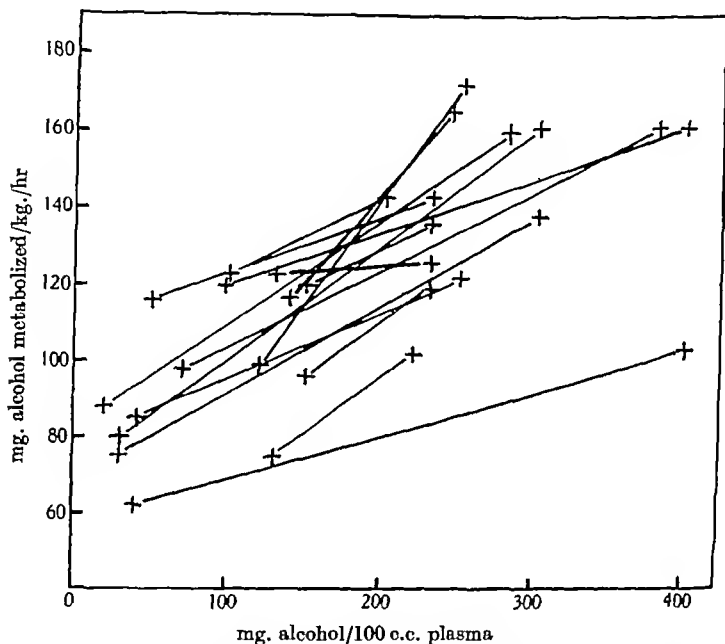


Fig. 2. The relationship between the metabolic rate of alcohol and its concentration in the plasma. Each pair of values represents the results obtained on one animal, by means of two separate injections of alcohol.

*Measurement of the metabolic rate over long periods following a single injection of alcohol.* The observations of Haggard & Greenberg [1934], of a 17 % decrease in metabolic rate per hour, could not be confirmed. There was a gradual reduction in the value of  $\beta$  with decreasing concentration of alcohol, as can be seen from the results summarized in Table I, but this was so small that over any period of 2-3 hr. the rate of decrease in plasma alcohol concentration appeared to be constant. The change of metabolic rate with changing concentration of alcohol is of the same order of magnitude as that observed in the experiments quoted above. The last experiment listed in Table I demonstrates the greater degree of change of metabolic rate with changing concentration of alcohol at a low plasma alcohol concentration.

TABLE I. Relation of metabolic rate of alcohol to its concentration in the plasma, following a single injection

Exp.	r	Time hr. after end of injection	Alcohol concentration in plasma mg./100 c.c.	$\beta$ mg./c.c./min.	Metabolic rate mg./kg./hr.
Dog 11.5 kg.	0.585	1-6	200-300	0.00315	110
		6-12	100-200	0.0026	90
		12-16	40-100	0.00245	84
		16-18	20-40	0.00205	70
Cat 3.75 kg.	0.625	1-5	220-290	0.0031	118
		5-8	170-220	0.00285	108
Cat 2.25 kg.	0.74	1-2	280-310	0.0044	196
		2-6	210-280	0.0033	147
		6-10	150-210	0.00245	109
Cat 2.85 kg.	0.58	1-3	30-60	0.00275	96
		3-4	15-30	0.00215	75
		4-5	5-15	0.0015	52

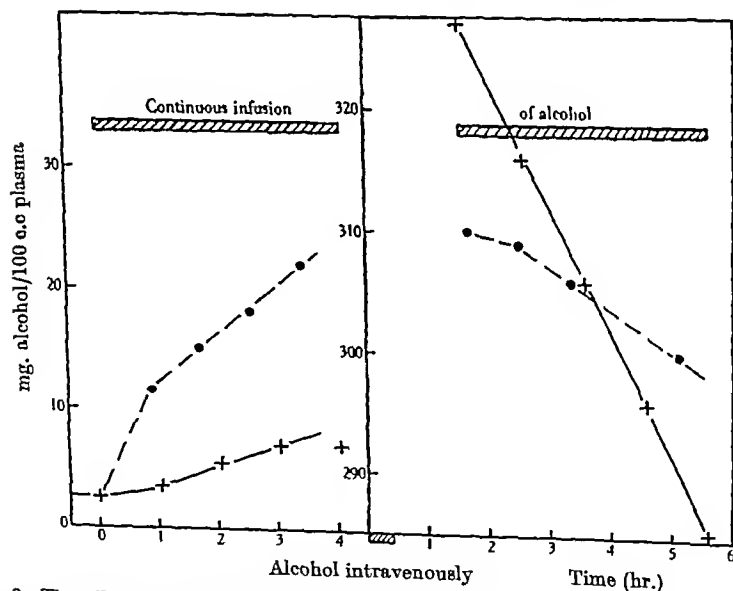


Fig. 3. The effect of a constant intravenous infusion of alcohol at low and at high blood alcohol concentrations. + — + = infusion at a rate of 270 mg./kg./hr. into a 3.25 kg. cat. • — • — • = infusion at a rate of 455 mg./kg./hr. into a 3.1 kg. cat. The increased concentration of alcohol shown in the right half of the figure was attained by the intravenous injection of 7.2 g. alcohol in the first experiment and 7.35 g. in the second.

*Comparison of the metabolic rate at widely varying concentrations by constant infusion of alcohol.* The results of two of the three experiments performed are shown in Fig. 3. In the first half of each experiment, alcohol was infused at a constant rate for some hours. An injection of

alcohol was then given in order to establish a high concentration in the body, and when equilibrium had been established, the infusion was continued at the same rate as before.

The infusion was given intravenously, from a 20 c.c. syringe operated by a screw driven by a geared motor. In the first experiment, a 7 % solution of alcohol was delivered at a rate of 3.65 c.c./hr.; in the second experiment, a 8.3 % solution at 5.5 c.c./hr.

It can be seen from Fig. 3 that in both experiments the metabolic rate at the high concentration of alcohol was greater than the infusion rate and less at the low concentration. Approximate values of  $r$  obtained from the second half of each experiment, 0.6 and 0.65 respectively, enable the actual metabolic rates to be calculated. They were as follows:

Exp.	Alcohol concentration in plasma mg./100 c.c.	Metabolic rate of alcohol mg./kg./hr.
1	2.5-7.5	70
	300	140
2	2.5-11.5	75
	11.5-22	120
	300	165

It is clear that at 0 mg./100 c.c. the metabolic rate must be 0, but above this value it rises very rapidly until a concentration of *ca.* 10 mg./100 c.c. is reached, and thereafter more slowly. At the lowest blood alcohol concentrations investigated the metabolic rate was already 70-75 mg./kg./hr., and it seems likely that the rate is limited up to these concentrations by the rate of blood flow through the liver. If, for example, the whole of the alcohol content of each unit of blood were removed by the liver, then the observed metabolic rate of 70 mg./kg./hr. would be obtained at 10 mg./100 c.c. by a blood flow through the liver of 37 c.c./min.—a reasonable figure for a cat [McMichael, 1938].

The question arises as to whether the relationship between metabolic rate and alcohol concentration invalidates the assessment of  $r$ , which is obtained by assuming a linear rate of decrease in blood alcohol concentration during the first few hours of an experiment, whereas in fact a much higher concentration exists during the first hour following an intravenous injection than during the subsequent period. It is probable that the metabolic rate is faster during this time and that the initial concentration obtained by extrapolation of the blood alcohol curve to zero time is fictitiously low. Conversely, when alcohol is ingested, its concentration in the body before absorption is complete and equilibrium attained is lower than in the later stages, and the assessed initial concentration fictitiously high. The error involved is, however, likely to be of small magnitude, and experiment shows it to be of no practical importance. In two animals, one dose of alcohol was given by intestine and the other intravenously; in one case,  $r$  was identical for the two injections (0.68), and in the other, values of 0.64 and 0.62 were obtained.

In all of the foregoing experiments, the effect of concentration of alcohol on its metabolic rate has been studied in individual animals only. When the metabolic rates of different animals were compared, however, it became apparent that no clear-cut relationship between metabolic rate and alcohol concentration could be demonstrated statistically. This is scarcely surprising, in view of the wide variation in metabolic rate encountered in different animals, as shown in Fig. 2. The relationship observed in any one animal was masked by some factor or factors varying in different animals, of which one was the actual body weight. The metabolic rate of alcohol tended to be lower in the larger animals.

### *Liver*

A rather clearer picture was obtained when the results were expressed in terms of body surface instead of body weight, but this correction failed to bring into line with the rest certain animals having very small livers. When the results were expressed in terms of liver weight, however, the quantitative relationship between metabolic rate and alcohol concentration was apparent. The first series of cats used (London) ranged in weight from 1.75 to 4.5 kg., and their livers from 1.4 to 3.7 % of the body weight, with an average value of  $2.3 \pm 0.1$  %<sup>1</sup> (16). In general, the larger animals had a smaller proportion of liver than the smaller animals, but a definite relationship could only have been established if a much larger group of animals had been studied.

In a second series of cats used (Cardiff), in which the body weight ranged from 2.0-3.1 kg., the liver weight varied between 3.0 and 4.25 % of the body weight, with an average value of  $3.4 \pm 0.1$  % (11). This value is significantly higher than that of the London cats, and the factors concerned in producing such a difference are a matter of conjecture only. But the difference itself has served to emphasize the fact that the metabolic rate of alcohol is correlated more closely with liver weight than with body weight. All the results obtained on these normal cats and on two dogs are shown in Fig. 4, together with those of two cats suffering from jaundice.

The lower rate of alcohol metabolism in animals suffering from jaundice is not surprising. In the experiments quoted in Fig. 4 the liver was of normal size, and the metabolic rate of alcohol was abnormally low by any criterion—liver weight, body weight or body surface. A similar result has been obtained by Erwtelman & Heeres [1933] in human subjects, in both acute and chronic liver damage and by Oelkers [1938] in rabbits suffering from experimental liver damage.

<sup>1</sup> S.E. of mean is given here and elsewhere in the paper.



In experiments on intact animals and man it is obviously impossible in our present state of knowledge to express results of alcohol metabolism in terms of liver weight, and until some method is found for assessing liver weight *in vivo*, it would seem as accurate to retain the body weight criterion as to express the results in terms of body surface.

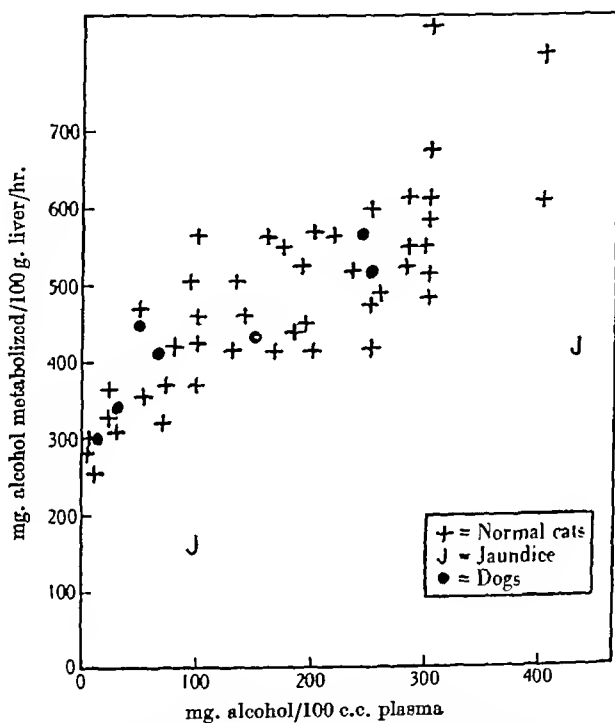


Fig. 4. The relationship between metabolic rate of alcohol per unit liver weight, and plasma alcohol concentration.

### "Tolerance"

An attempt was made to induce "tolerance" to alcohol in sixteen cats. The sole source of food was milk containing 5 % alcohol (by weight) which was left overnight with the animals. The alcohol concentration had fallen to about 4 % by the morning, and the alcohol consumed was calculated on an average figure of 4.5 % in the milk drunk. Control animals kept in captivity under the same conditions were fed on milk with occasional scraps of meat. Nine of the sixteen animals refused to touch the drink and after steadily losing weight for a few days were returned to normal diet. Three of these showed either jaundice or

obviously diseased liver when used later for experiment, as did also three of the remaining seven which had consumed alcohol in varying amount. Of these seven, the highest consumption reached was 3.5 g./kg./day—i.e. the equivalent of a bottle of whisky a day to a man—for 40 days.

Of the seven animals which consumed alcohol in varying amount (0.5–3.5 g./kg./day) and for varying times (13–52 days), two maintained their body weight (one of which was the heaviest drinker of them all), but the remaining five lost from 5 to 28 % of their body weight during the course of the alcohol feeding. Of the three of these found to be suffering from jaundice at the time of experiment, two had been light drinkers (0.5 g./kg./day). The average liver weight of the seven was  $3.4 \pm 0.3$  % of the body weight. These animals were used under the same conditions as the controls having an average liver weight of  $2.3 \pm 0.1$  % of the body weight; thus, there would appear to be a significant increase in liver weight as a result of the alcohol feeding. In view of the fact that a further series of control animals used in other circumstances also had an average liver weight of  $3.4 \pm 0.1$  %, however, this conclusion must be drawn with reserve.

The increased percentage liver weight of the "tolerant" animals was not correlated with loss of body weight: the heavy drinker who maintained his body weight unchanged had one of the largest livers (4 %).

When the metabolic rate of alcohol was determined in these seven "tolerant" animals, no significant difference from that in the control animals was observed, if the results were expressed in terms of body weight. The metabolic rate per unit of liver weight was significantly lower, however, as can be seen from the statistical analysis given in

TABLE II. Relationship between metabolic rate of alcohol (per 100 g. liver) and its concentration in the plasma in (A) normal cats and (B) cats previously fed with alcohol

Alcohol concentration in plasma mg./100 c.c.	Metabolic rate of alcohol mg./100 g. liver/hr.	
	A	B
ca. 10	$280 \pm 22$ (2)	140 (1)
ca. 20	$339 \pm 10$ (4)	163 (1)
50–100	$403 \pm 25$ (8)	$265 \pm 50$ (2)
100–200	$460 \pm 20$ (9)	$374 \pm 30$ (3)
200–300	$520 \pm 20$ (12)	$366 \pm 35$ (5)
> 300	$650 \pm 35$ (7)	

Table II. The "tolerant" animals appear to metabolize alcohol at 50-80 % the rate of the normal animals with the same size of liver, and the results suggest that in the "tolerant" animals an upper metabolic limit is reached which cannot be increased by further increase in alcohol concentration. Additional evidence for the soundness of this conclusion is afforded by the second series of control animals. These had the same average liver weight as the "tolerant" animals, and the metabolic rate of alcohol in the latter was lower than the normal whether expressed in terms of liver weight or body weight.

### *Anaesthetic*

Various observations indicated that deep nembutal anaesthesia reduced the metabolic rate of alcohol and suggested the possibility that even the light degree of anaesthesia normally used in these experiments might affect the results. No information has been obtained from the literature in regard to the behaviour of alcohol in cats without anaesthetic, but much work has been done on unanaesthetized dogs and it seemed of interest to compare with them the animals used in this series under nembutal. In Table III are some results of Mellanby [1919] on two dogs

TABLE III. The metabolic rate of alcohol in animals under nembutal anaesthesia compared with those of other workers without anaesthetic. Figures in brackets indicate the number of observations

Alcohol concentration in plasma mg./100 c.c.	Metabolic rate mg./kg./hr.						
	No anaesthetic			Nembutal			
				Dogs		Cats*	
	Mellanby (2 dogs)	Widmark (6 dogs)	Haggard & Greenberg (4 dogs)	1	2	A (11)	B (10)
10-20	—	—	—	—	76	81 (2)	120
50-100	—	104 (12)	—	86	116	109 (4)	140 (2)
100-200	120	120 (2)	230, 290	92	—	114 (3)	150 (7)
200-300	150	—	600	110	143	137 (7)	162 (2)
300-400	200 (2)	—	510	—	—	161 (2)	—

\* The cats were separated into two groups by liver weight: group A, liver weight 2-3 % of body weight: group B, 3-4 %.

and of Widmark [1933a] on six dogs, together with those of Haggard & Greenberg [1934] on four dogs. Of the two dogs under nembutal, the first had a liver weight of 2.1 % of the body weight and the second, one

of 2.5 %. The cats quoted are divided into two groups according to liver weight, those with livers 2-3 and 3-4 % of the body weight respectively. It is clear that this variable factor is of far greater importance in affecting the metabolic rate of alcohol than any possible inhibitory action of nembutal, and one may conclude that this anaesthetic, in the dosage used, has little action. The cause of the wide variation of the results of Haggard & Greenberg from those of other workers is obscure.

### *Alanine*

It is a matter of common observation that when alcohol is ingested with food, its effects on the nervous system are not so pronounced as when the same dose is taken on an empty stomach. That this effect is associated with a reduced concentration of alcohol in the blood was first shown by Mellanby [1919]. His results were amplified by Southgate [1925], who found that a meal of bread and milk was more efficacious than one of olive oil in reducing the concentration of alcohol in the blood after a given dose. Widmark [1933*b*, 1934] found that amino acids were more potent in this respect and fatty acids less so; carbohydrate and fat had no such effect. This action of foodstuffs cannot be attributed solely to their effect in delaying absorption of the alcohol since the blood-alcohol curve in these conditions stays consistently at a lower level than that following absorption of the same amount of alcohol from an empty stomach. Moreover, Le Breton [1934] finds that the total alcohol content of the body disappears more rapidly in these circumstances. The simplest explanation of this lower blood-alcohol curve, namely a combination of delayed absorption and increased rate of metabolism due to the specific dynamic action of the foodstuffs, has received scant attention. The results of Leloir & Muñoz [1938] suggest that this latter factor may be of importance, for they found the metabolism of alcohol in liver slices to be increased by the addition of both alanine and pyruvic acid. Widmark, however, insists that under the action of amino acids, and to a less extent of fatty acids, some of the alcohol "disappears". He postulates an ester formation between the two, but has, as yet, been unable to identify such a compound in the blood or tissues. It seemed possible that a reinvestigation of the problem under different experimental conditions might yield further information, and experiments were designed, therefore, to test the effect of alanine under conditions in which control values of  $r$  and  $\beta$  could be obtained in the same experiment.

It became clear at an early stage in the work that the action of a single dose of alanine on alcohol metabolism was too short-lived to yield any conclusive evidence as to its mode of action, and in the later experiments an intravenous infusion of alanine was continued over several hours. Under these conditions, the evidence was clear-cut. The value of  $r$  was unchanged and that of  $\beta$  considerably increased, remaining at the higher figure so long as the infusion was continued. Most of the experiments were of the type illustrated in Fig. 1, the alanine being given with either the first or second dose of alcohol. A summary of all the results obtained is given in Table IV. In several experiments, alanine

TABLE IV. The effect of alanine on the metabolic rate of alcohol

Weight of cat kg.	Total alanine injected g.	$r$	$\beta$ mg./c.c./min.	Plasma alcohol concentration mg./100 c.c.
2.3	0	0.52	0.00345	250-300
	2	—	0.00465	200-250
2.5	2	0.605	0.00435	40-80
	0	0.59	0.0037	110-230
3.12	2	0.65	0.0032	80-130
	0	0.64	0.0032	130-180
2.26	0	0.59	0.00415	70-130
	5	0.59	0.0079	130-170
2.0	0	?	0.0048	150-200
	5	0.61	0.006	150-200
2.2	5	0.62	0.0053	50-130
	0	0.62	0.00375	80-120
2.8	0	0.645	0.0035	80-130
	5	<0.68	0.00455	110-140
2.28	0	0.64	0.00425	100-150
	6	0.65	0.0059	80-150

was given with the smaller dose of alcohol; its effect in increasing the metabolic rate was so great as to over-ride the opposing concentration effect in all but one experiment, in which a single small dose of alanine had been given to a rather large cat. In the first experiment listed, a single dose of alanine was given without further alcohol; the value of  $\beta$  increased for the next  $1\frac{1}{2}$  hr. and returned to the original value for the succeeding hour. This observation is not in accord with Widmark's conclusion [1933c] that the "disappearance" of alcohol occurs only if the protein or amino acid is given either with or before the alcohol. His conclusion, however, was based on an experiment in which a protein meal was given while the blood-alcohol concentration was decreasing, and its specific dynamic action would not have occurred until the concentration of blood alcohol was only about 20 mg./100 c.c., the region in which its metabolic rate falls away so markedly.

Further evidence that Widmark's own experiments do not necessitate the "ester-formation" hypothesis was obtained by comparing an experiment performed in the same way as his own with one in which a constant infusion of alanine was given. In each animal, an initial dose of alcohol was injected into the stomach, and the blood-alcohol curve followed in order to obtain a control value of  $r$ . Then, in the one cat (2.26 kg.) analogous to Widmark's experiments, a dose of alanine (5 g.) was injected into the stomach shortly followed by a second dose of alcohol calculated to bring the blood-alcohol concentration back to that obtaining during the control period. For ease of presentation, the two blood-alcohol curves have been superimposed (Fig. 5A).

In the first half of the experiment, 2.48 g. alcohol were given;  $r$  was 0.59,  $\beta$  0.00415. The second dose (1.8 g.) raised the concentration to a rather higher value than that resulting from the first dose,  $C_0$  being 206 mg./100 c.c. as compared with 186 mg./100 c.c. In Fig. 5A, therefore, 20 mg./100 c.c. has been subtracted from each point on the second curve. A similar adjustment of 15 mg./100 c.c. has been made in Fig. 5B, since the  $C_0$  value following the second dose (1.85 g.) was 204 mg./100 c.c. as compared with 189 mg./100 c.c. following the first dose (2.78 g.).

If the early part of each curve in Fig. 5A is ignored (their differences will be considered later), the results are the same as those obtained by Widmark. That is to say, when  $\beta$  has attained a steady value, the blood-alcohol curve following the alanine administration is parallel with, but lower than, the control curve. Either some alcohol has "disappeared", or the value of  $r$  has increased—or the metabolic rate of alcohol has been faster during the first hour or so after the administration of the alanine.

In the second cat, the same procedure was followed (2 g. alanine to a 2.28 kg. cat), but alanine was infused intravenously (1 g. in 5.5 c.c./hr.) throughout the second period (saline having been infused at the same rate during the control period). There is no longer any "disappearance" of alcohol (Fig. 5B) in spite of the increased amount of alanine present, but a definite increase in the value of  $\beta$ ,  $r$  remaining unchanged. The simplest explanation of these results is that amino acids increase the metabolic rate of alcohol. Widmark's "ester-formation" hypothesis is untenable in its present form, although the possibility that such formation occurs normally in the body cannot be ignored. The alcohol disappearing from the blood would then be due to a combination of ester formation in the tissues together with oxidation, and the addition of amino acids to the body might well hasten both processes. If this were so, however, the whole method of assessment of metabolic rate of alcohol from analysis

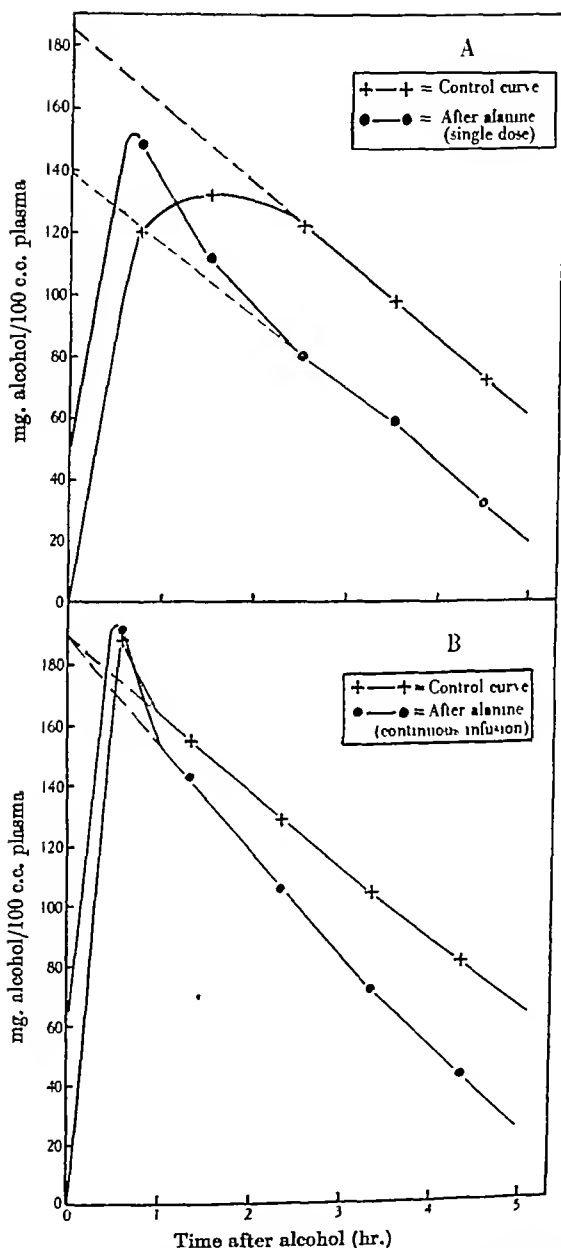


Fig. 5. A. Comparison of plasma alcohol concentration following a dose of alcohol into the stomach (+—+), and a dose of alcohol combined with a single dose of alanine (•—•). B. A similar comparison, with a constant intravenous infusion of alanine continued throughout the period following the single dose (•—•).

of blood alcohol concentration falls to the ground, and until definite evidence of the existence of such esters is forthcoming, it would seem justifiable to adhere to the simpler hypothesis. All the published work concerning the action of foodstuffs on alcohol metabolism can be interpreted in this way if the relative time relations of delayed absorption and specific dynamic action are taken into account.

In both experiments (Figs. 5A, 5B) a volume of water was given with the first dose of alcohol equal to that of the alanine solution given with the second dose. In spite of this, the absorption of the second dose in Fig. 5A was much more rapid than that of the first, a fact noted by Mellanby. In Fig. 5B there is very little difference in the rate of absorption of the two doses, and the rate of absorption is greater than the rate of diffusion of alcohol from the blood into the tissues. This commonly occurs when alcohol is injected directly into the small intestine, and it seems probable that in this experiment the pylorus remained open after the injections, allowing the fluid to pass rapidly into the duodenum.

### SUMMARY

1. The metabolic rate of alcohol in cats was found to be directly dependent on the concentration of alcohol in the body, increasing about 30 % for every 100 mg./100 c.c. increase in plasma alcohol concentration. This relationship, although apparent in each individual animal, could be demonstrated statistically on all the animals used only if the metabolic rate was expressed in terms of liver weight, rather than body weight or body surface.

2. The metabolic rate of alcohol per unit liver weight in animals previously fed with alcohol was significantly smaller than in normal animals.

3. Comparison of results obtained on animals under light nembutal anaesthesia with those of other workers on unanaesthetized animals shows that this anaesthetic has little, if any, inhibitory action on the metabolic rate of alcohol.

4. The metabolic rate of alcohol was increased by addition of alanine to the body,  $r$  remaining unaffected and  $\beta$  being increased. Widmark's hypothesis of the "disappearance" of alcohol by ester formation in these circumstances is shown to be unnecessary.

My sincere thanks are due to Prof. Graham Brown for giving me facilities to complete this work in his laboratory.



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## THE RESULTS OF ADRENALECTOMY IN THE PREGNANT ALBINO RAT

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THE possibility of some interaction between the adrenals and the sex glands and genital tract during pregnancy has been suggested by several observers. Such interaction may be considered from two different aspects. First, can pregnancy do anything to support or replace adrenal function and, secondly, do the adrenals make any essential contribution to the maintenance and normal progress of pregnancy?

Rogoff & Stewart [1927] reported prolongation of survival in twelve of seventeen bitches adrenalectomized in various stages of pregnancy, and this has been confirmed in the same species by Billmann & Engel [1939]. Firor & Grollman [1933] reported similar findings in the albino rat, in contradiction to the observations of Carr [1931]. Numerous investigators have agreed that there is no improvement in the survival of cats adrenalectomized during pregnancy [Corey, 1928; Rogoff & Stewart, 1929; Grădinescu, Santa & Lucan-Ionescu, 1935].

The available evidence on the second question is conflicting. Adrenalectomy in pregnant bitches does not seem to interfere with the progress of pregnancy nor with parturition [Rogoff & Stewart, 1927; Billmann & Engel, 1939]. In pregnant cats in most cases the operation is followed by abortion or resorption of the fetuses [Corey, 1928; Rogoff & Stewart, 1929; Britton & Kline, 1936]. Lewis [1923] and Ingle & Fisher [1938] reported no change in the course of pregnancy after adrenalectomy in the rat, although Britton & Kline [1936] stated that the operation in this species commonly resulted in abortion.

In this paper an account is given of an experimental examination of these questions.

<sup>1</sup> Hilda and Ronald Poulton Research Student.

## METHODS

Albino rats, bred from Glaxo stock, were used. Daily vaginal smears were examined to check uniformity before and during the experimental period. The date of pregnancy was established by the presence of sperm in the full oestrus smear and confirmed by the non-appearance of the oestrous cycle.

Under ether anaesthesia the adrenals were exposed at one operation through separate incisions in the flanks. The glands were removed with a surrounding veil of peritoneum and connective tissue, care being taken to avoid rupture of the capsule and to control bleeding. When salt was given it was administered by replacing the drinking water with normal saline.

For the estimation of the weight of suprarenals of the newly born, the young were killed on the day of delivery, the abdomen opened and the animals fixed in Bouin solution for 24 hr. The adrenals were then shelled out, dried between filter-papers and weighed.

## RESULTS

In view of the fact that the results of adrenalectomy had not previously been studied in this colony of rats preliminary experiments were performed to show the effect of sex, weight and salt treatment on survival after the operation.

*The effect of sex, weight and salt treatment on the survival  
of adrenalectomized animals*

A comparison was made between the survival periods after adrenalectomy of a group of fifteen female rats weighing 130–150 g. and a group of fifteen male animals of corresponding weights. The latter was also compared with another group of fifteen males weighing over 200 g. The results are given in Table I.

TABLE I. The effect of weight, sex and salt treatment on the survival of adrenalectomized animals

No. of animals	Sex	Weight (g.)	Treatment	Period of survival days
15	Female	130–150	None	$7.5 \pm 0.29$
15	Male	130–150	None	$8.9 \pm 0.36$
15	Male	>200	None	$11.7 \pm 0.57$
15	Male	>200	Salt	$28.9 \pm 2.24^*$ (7 animals)

\* Three animals, salt removed at 24 days; five animals, salt removed within 7 days of salt removal in each case.

As the table indicates the mean period of survival of the males was slightly but significantly greater than that of the females of corresponding weights. The table also shows that male animals weighing over 200 g. lived considerably longer than animals of the same sex weighing 130-150 g.

A group of fifteen male rats weighing over 200 g. was adrenalectomized and maintained on salt. For seven of these animals the mean period of survival was 28.9 days. The salt was taken from three animals of the group on the 24th day after the operation and from five animals on the 36th day. These eight animals died within 7 days of its removal.

### *Adrenalectomy during pregnancy*

Fifteen animals weighing 130-150 g. were adrenalectomized between the 1st and 4th days of pregnancy, and a further group of fifteen animals of the same weight between the 7th and 9th days. The results are given in Table II and the mean survival periods are compared with that of the control group of non-pregnant female animals of the same weight.

TABLE II. Results of adrenalectomy during pregnancy

No. of animals	Weight g.	Pregnant or non-pregnant	Nature of operation	Date of operation in days of pregnancy	Effect on pregnancy	Period of survival days
15	130-150	Non-pregnant	Bilateral adrenalectomy	—	—	$7.5 \pm 0.29$
15	130-150	Pregnant	"	1st-4th	Abortion or no implantation	$9.9 \pm 0.27$
15	130-150	"	"	7th-9th	Abortion or resorption of foetus	$11.6 \pm 0.87$
10	>200	"	"	7th-9th	"	$17.6 \pm 0.9^*$
5	175-200	"	Unilateral and dummy operation	7th-15th	None	Indefinitely

\* One animal survived 44 days and delivered and reared a normal litter.

The pregnant animals lived significantly longer after adrenalectomy than did the controls.

None of these animals survived to term. It was also observed at autopsy that in all cases adrenalectomy had interfered with the progress of pregnancy. In the animals operated on between the 1st and 4th days the operation either prevented implantation or led to a subsequent abortion, for no sign of the pregnancy was found in the uterus either at autopsy or at occasional laparotomies some days previously. The operation in the animals 7-9 days pregnant resulted either in abortion or resorption of the foetuses.

Ingle & Fisher [1938] did not report any interference with pregnancy in rats adrenalectomized during pregnancy, and it was thought that this difference might be accounted for by the greater weights of the animals used by these workers. To verify this point a further ten animals weighing over 200 g. were adrenalectomized between the 7th and 9th days of pregnancy (see Table III). In nine animals the operation led to abortion or resorption of the foetuses. The single exception delivered and reared young, but as this animal survived 44 days after the operation it is probable that accessory cortical tissue was available to it.

To determine whether mere operative interference was responsible for the disturbance of pregnancy, dummy operations were performed between the 9th and 15th days of pregnancy in five rats. The right suprarenal and a portion of fat near the left suprarenal were removed. All these animals proceeded to term, delivered and reared normal young.

*Adrenalectomy in pregnant animals maintained on salt*

It has been stated that in the rat the usual effects of adrenalectomy on the sexual cycle [Kutz, McKeown & Selye, 1934] and on lactation [Gaunt & Tobin, 1936; Levenstein, 1937] can be prevented by administration of salt after the operation. In order to see whether this also

TABLE III. The results of adrenalectomy during pregnancy in animals (130-140 g.) maintained on salt

Animal	Date of operation in days of pregnancy	Day of return of oestrus	Delivery	Lactation	Survival
1	6	Day of delivery	Normal	Unsuccessful	Alive on salt treatment 2 months after operation
2	7	"	"	"	56 days
3	8	"	"	"	37 days
4	8	No return	"	"	52 days
5	9	Day of delivery	"	"	Alive on salt treatment 2 months after operation
6	7	"	"	"	"
7	9	No return	"	"	"
8	9	Day of delivery	1 dead foetus	No attempt	"
9	8	Day of delivery	Dead litter	"	43 days
10	8	4th day after operation	None	—	Alive on salt treatment 2 months after operation
11	9	5th day after operation	"	—	"
12	9	3rd day after operation	"	—	"
13	8	4th day after operation	"	—	"
14	7	4th day after operation	"	—	40 days
15	8	4th day after operation	"	—	20 days

applies to the pregnant animal fifteen females weighing 130–140 g. were adrenalectomized between the 6th and 9th days of pregnancy and maintained on salt. The results are given in Table III.

In six animals oestrus reappeared between the 3rd and 5th days after the operation, and subsequently at regular 4–5 days' intervals. Laparotomies showed that abortion must have occurred in these cases. Two of the remaining nine animals delivered dead litters at term, while in the other seven parturition was normal.

The young delivered by these seven animals were taken for examination of the adrenals, and were replaced by normal litters. In no instance was lactation successful. These mothers attempted to care for the young, but were unable to do so, for all the litters were found dead within 3 days of delivery. Normal oestrous cycles reappeared in the mothers within a few days of the death of the young.

Table IV gives a comparison between the weights of the adrenals of the young delivered of adrenalectomized mothers maintained on salt and the weights of the adrenals of young delivered of normal mothers.

TABLE IV A comparison between the weights of the adrenals of young delivered of adrenalectomized mothers maintained on salt and the weights of the adrenals of young delivered of normal mothers

Maternal history	No. of mothers	Average weight of mothers g.	Total no. of foetuses	<i>a</i>		<i>b</i>	Mean <i>b/a</i>
				Mean weight of young g.	Mean weight of adrenals of young mg.		
Normal pregnancies	8	176	54	5.4 ± 0.09	1.47 ± 0.03	0.272 ± 0.007	
Bilateral adrenalectomy 6th–9th day of pregnancy, maintained on salt	7	140	43	4.6 ± 0.08	1.31 ± 0.07	0.284 ± 0.016	

The mean weight of the adrenals was considerably less in the adrenalectomized group, as also was the mean foetal weight. The ratio of the suprarenal weight to weight of foetus showed no appreciable difference between the two groups.

*The survival of pregnant animals after the removal of the adrenals and foetuses*

The experiments described in the previous section suggest that the foetal adrenals are not responsible for the prolonged survival of animals adrenalectomized during pregnancy. This view finds further support in the following experiment.

Eight animals weighing 135–150 g. were operated on between the 9th and 11th days of pregnancy. Both adrenals were removed through

separate incisions in the flanks, and all the foetuses through a mid-line ventral incision, the placentae being left intact.

The mean survival period of these eight animals was  $17.4 \pm 2.44$  days. While there was greater variation in the survival of individual animals, the mean survival period of the group was significantly longer than that of non-pregnant adrenalectomized animals of corresponding weights. This increase in survival was at least as great as that observed in the pregnant, adrenalectomized animals in which the uterus was not interfered with.

### DISCUSSION

The results confirm the reports that pregnant rats live longer after adrenalectomy than do non-pregnant controls. Grollmann [1936] has suggested that the foetal adrenals provide the mother with cortical hormone, and in support of this view are the observations of Ingle & Fisher [1938] who found that the adrenals of newly-born rats were significantly heavier if the mothers were adrenalectomized at the 7th or 14th days of pregnancy. Our experiments do not support this view since:

(a) Survival was prolonged after adrenalectomy during pregnancy in spite of the fact that in every case abortion or resorption of the foetuses occurred.

(b) The foetuses of the young delivered of mothers adrenalectomized during pregnancy and maintained on salt were not heavier than those delivered of normal mothers.

(c) Pregnant animals from which the foetuses and adrenals were removed at the same operation showed the same improvement in survival as adrenalectomized pregnant animals in which the foetuses were left intact.

Evidence is available which suggests that the corpus luteum plays an important part in prolonging the lives of adrenalectomized animals [Emery & Schwabe, 1936; Swingle, Parkins, Taylor, Hays & Morrell, 1937; D'Amour & D'Amour, 1939; Collins, 1939]. In view of our results this appears to be a more likely explanation, and recently it has been shown that progesterone in adequate doses will for a time at least replace the cortical hormone [Gaunt & Hays, 1938, in ferrets; Corey, 1939, in cats; Gaunt, Nelson & Loomis, 1938; Bourne, 1939; Schwabe & Emery, 1939; Greene, Wells & Ivy, 1939; Fischer & Engle, 1939, in rats].

The fact that seven of the fifteen mothers, adrenalectomized between the 6th and 9th days of pregnancy and maintained on salt, delivered litters which were apparently normal does not suggest any essential contribution from the adrenal for the maintenance of pregnancy, at least

in the later stages. On the other hand, six animals aborted in spite of salt treatment, and resumed normal oestrous cycles. The control experiments make it unlikely that operative shock was a factor in these results. It is interesting to recall that there is evidence that ovulation and luteinization are not normal in adrenalectomized animals [Friedgood & Foster, 1937, 1938]. It is possible that the adrenal is essential in the earlier stages of pregnancy, and that the differences in our results are explained by the fact that all the operations were performed shortly before the middle of pregnancy.

#### SUMMARY

1. Adrenalectomy between the 1st and 4th days of pregnancy in albino rats either prevented implantation or led to abortion; between the 7th and 9th day adrenalectomy led to abortion or resorption.

2. A salt diet prevented in part the effects of adrenalectomy as nine out of fifteen adrenalectomized mothers delivered litters (two dead) but were unable to rear young. The remainder aborted and resumed regular oestrous cycles.

3. The adrenals of the young of salt-treated, adrenalectomized mothers did not differ in weight from controls.

4. In pregnant animals simultaneous removal of foetuses and adrenals was followed by the same increased survival period as after adrenalectomy alone.

5. It is concluded that the observed increase in survival period of rats adrenalectomized during pregnancy cannot be due to the activity of the foetal adrenals.

One of us, W. R. S., is indebted to the Government Grant Committee of the Royal Society for a grant towards the animal expenses of this work.

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## EFFECTS OF SYMPATHOMIMETIC AMINES ON PERFUSED BLOOD VESSELS<sup>1</sup>

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PREVIOUS studies of the actions of sympathomimetic amines in pathological states of the circulation [Tainter, Footer & Hanzlik, 1939; Crismon, C. A. & Tainter, 1939] have indicated the desirability of knowing the relative quantitative effects of these agents on different elements of the circulatory system, in order that they might be used according to some specific seat of action. Certain data for such information were available from other published reports from this laboratory on the potency of these compounds for the intact normal circulation, and for the isolated heart, in a heart-lung preparation [Crismon, J. M. & Tainter, 1938]. This paper presents results with the same amines on the blood vessels of the cat's perfused hindleg, as the first of a series of studies of their vasomotor effects. In the hope of being able to account for the magnitude of the blood-pressure reactions in the various studies, there have been considered also the various conditions for reactivity of the vessels in the excised legs. The results obtained are believed to be of importance not only for the choice of these amines to be used in pathological states, but also in understanding the humoral transmission mechanisms in the blood vessels.

### METHODS

In cats, anaesthetized with sodium pentobarbital, the femoral artery was cannulated and the blood washed out of a hindleg with physiological salt solution (0.85% NaCl). This leg was amputated through the hip

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joint and transferred to a glass funnel kept warm by a hot-water jacket. The inflow was through the cannula in the femoral artery, with the perfusion fluid flowing through coiled glass tubing in a constant temperature water bath at  $37.8^{\circ}\text{C}$ . The femoral vein was left open, uncannulated, so that the outflow ran off the leg into the funnel, and was collected in graduated cylinders. Special attention was given to eliminating all metal from contact with the perfusion fluid, since our experience agreed with that of Beland, Moe & Visscher [1938], that enough metal could be given off to the fluid to poison the perfused leg within a comparatively short time.

In the majority of legs, pressure for the perfusion was supplied by compressed air held constant by a mercury escape valve. The pressure was so regulated that a flow between 20 and 40 c.c./min. was secured, which required between 40 and 80 mm. Hg pressure when Locke's solution was perfused. Injections of the amines were made through the rubber tubing at the cannula in a constant volume of 1 c.c. of fluid. Control injections of the perfusion fluid alone did not produce demonstrable changes in the outflow, so that the mechanical effect of the injection itself was negligible. Vasoconstriction was indicated by a slower rate of outflow, and vasodilatation by an increase. The responses appeared during the first minute after injection, became maximum in from 2 to 5 min., and lasted generally from 5 to 10 min. Recovery to the pre-injection level was almost invariable, except after very large doses of the less active amines. Under ordinary conditions, good responses were obtained for over 3 hr., and during this time closely reproducible effects were obtained with repeated doses of those amines whose actions were not complicated by tachyphylaxis.

The general procedure was to inject a dose of adrenaline, which would give a definite but not maximum vasoconstriction, usually about 35%, and then to match the change, if possible, with a suitable dose of the amine in question. The injections were alternated with varying doses until a bracket of the responses to the two agents was achieved from which their relative activity could be computed. A given leg was used until signs of loss of reactivity, or oedema, developed. Each leg served for one determination on about three amines, and from three to nineteen individual legs were used for each compound under each set of conditions, the average number being about ten. Less than five legs were used only where there was unusually close agreement between them, or when there was no significant vasoconstrictor action. Diminished responses were obtained with injections of ephedrine and other phenyl ring compounds

made it difficult, if not impossible, to get more than a single acceptable vasomotor response to these agents. Therefore, these were injected only once in a given preparation.

### *Computation of results*

Inasmuch as this study was directed primarily at determining the relative potency of the various amines, individual variations would decrease the reliability of the results, unless the number of experiments were increased to somewhat impracticable levels. In bioassays this difficulty is largely obviated by using a standard of reference with which the unknown drug is compared, the average ratio of activity between the two being taken as the measure of the potency of the unknown drug. This was the method adopted in this study, using adrenaline as the reference standard. The choice of adrenaline for this purpose was particularly fitting in view of its role in the responses to the amines described below.

Comparisons of the responses were made in terms of both the absolute and the percentage maximum decreases in outflow, and of the absolute and percentage decreases in total flow in fifty consecutive legs. Since the variabilities by these various methods of calculation were practically the same, the maximum percentage decreases in the perfusion flow were finally used, as in our previous studies of these amines. From these values the ratio of potency of the amine to adrenaline was calculated by linear interpolation, using the bracketing responses. Its average value was computed from a series of determinations, together with the standard error of the mean, to indicate the probable variability. The standard-error calculations involved the possibly hazardous assumption that the individual values were normally distributed, an assumption difficult to prove in such limited series. The coefficient of skewness in ten different series of observations made on a total of 174 legs had a median value of 1.8, a value greater than was desirable, but not definitely establishing a departure from normality so great as to preclude application of the usual formulae. Since almost all the skewness resulted from abnormally high values, it is possible that the distribution of the logarithms of the ratios would have been more normal.

An alternative method of calculating the relative activities would be to derive equations expressing the relationship between dose and effect. The ratios of the constants of these equations to those of adrenaline would express the relative activities of the two agents. In the most suitable experiments for this purpose, such calculations gave values with

no smaller variabilities than were obtained from the simple linear interpolation method described above. Therefore this was adopted as being adequate under the restricted conditions of this study.

## RESULTS

### *Amines in Locke's fluid*

Perfusions of the amines were made first with the classical Locke's fluid of the following composition: sodium chloride, 0.92%; potassium chloride, 0.042%; calcium chloride, 0.012%; sodium bicarbonate, 0.015%; dextrose, 0.1%.

The activities observed are summarized in Table I, which shows the mean dosage ratio of each amine to adrenaline for equal vasoconstrictions,

TABLE I. Influence of the perfusion fluid on the vasoconstrictor ratios to adrenaline shown by various sympathomimetic amines when perfused through cat's leg vessels at a constant head of pressure

Amines	Locke perfusion fluid			Blood perfusion fluid			Change of activity in blood	
	No. of cats	Ratio to adrenaline		No. of cats	Ratio to adrenaline		Mean	Median
		Mean $\pm$ s.d.	Median		Mean $\pm$ s.d.	Median		
Dihydroxy-phenyl compounds:								
Arterenol	7	1.85 $\pm$ 0.41	2.1	3	1.12 $\pm$ 0.05	1.09	1.7	1.9
3-4-Dioxyephedrine	6	21.9 $\pm$ 2.3	21.3	7	31.4 $\pm$ 4.77	26.7	0.70	0.80
Cobefrine	10	9.9 $\pm$ 1.3	8.6	19	3.2 $\pm$ 0.37	2.7	3.1	3.2
Ethylorsuprenenol	10	1273 $\pm$ 190	1086	3	238 $\pm$ 15.3	250	5.4	4.3
Epinine	9	6.1 $\pm$ 0.75	6.6	3	7.2 $\pm$ 0.05	7.1	0.85	0.93
Metahydroxy-phenyl compounds:								
1-m-Oxyephedrine	9	294 $\pm$ 47.3	292	10	148 $\pm$ 31.8	93.9	2.0	3.1
Neosynephrine	13	22.6 $\pm$ 4.3	22.2	8	11.1 $\pm$ 0.98	9.7	2.0	2.3
m-Oxynorephedrine	8	191 $\pm$ 27.2	185	8	48.2 $\pm$ 7.0	45.2	4.0	4.1
Para- and ortho-hydroxy-phenyl compounds:								
Paredrine	14	7232 $\pm$ 1557	6020	9	33.4 $\pm$ 11.5	26.7	214	225
Tyramine	14	5017 $\pm$ 1470	3334	14	28.9 $\pm$ 5.1	27.6	174	121
o-Oxyephedrine	8	Mainly dilator	—	5	11.2 $\pm$ 4.68	4	$\infty$	$\infty$
Phenyl compounds:								
Ephedrine	15	31,100 $\pm$ 9044	5435	8	71.6 $\pm$ 10.5	68.6	434	70.2
Benzedrine	8	60,200 $\pm$ 15,900	48,076	6	Mainly dilator	—	—	—
Phenylethylamine	8	66.9 $\pm$ 17.0	67.1	6	6.1 $\pm$ 0.86	7.2	10.7	9.3
Phenylethanolamine	8	74.2 $\pm$ 38.4	25.0	9	3.75 $\pm$ 0.51	3.51	19.8	7.1
Propadrine	7	32,573 $\pm$ 10,500	29,412	10	36.5 $\pm$ 6.24	29.2	892	1007
Diphenylethylamine	5	63,071 $\pm$ 10,040	58,333	4	Mainly dilator	—	—	—

and the standard error of the mean. The median is included to indicate the normality of the distribution.

It was observed that the catechol derivatives, arterenol, 3-4-dioxyephedrine, cobefrine and epinine had potency of the same order of

magnitude for vasoconstriction of perfused vessels as for pressor actions on the intact circulation. Ethyl-norsuprarenin, although a catechol, was in a different category, since its actions are quite complicated, and include important dilator effects, due presumably to its long side chain [Cameron, Crismon, Whitsell & Tainter, 1938]. The metahydroxy-phenyl derivatives, 1-*m*-oxyephedrine, neosynephrine and *m*-oxynorephedrine also showed vasoconstrictor powers consistent with previous results under other conditions.

In marked contrast to the effects of these compounds were the responses to the ortho- and para-hydroxy-phenyl and unsubstituted-phenyl ring derivatives. With these the reactions were practically completely lacking. In order to get any response at all, the concentrations and volumes injected had to be raised to the point where possibly non-specific reactions to hypertonicity or acidity occurred, and even these were minimal, unpredictable, and showed little relationship between size of dose and effect. The size of the ratios to adrenaline obtained under these conditions obviously lacked quantitative significance, except to indicate an almost complete absence of vasoconstrictor power. From the ratios of 66 and 74, phenylethylamine and phenylethanolamine might not be regarded as belonging to this group of compounds. However, they were affected by the conditions described below in the same way as the other phenyl derivatives.

This lack of response of the perfused blood vessels might have been interpreted as indicating that these compounds did not possess direct vasoconstrictor power and that they produced their pressor action *in vivo* through cardiac, central, or other mechanisms. However, before such an explanation could be accepted, it was thought desirable to repeat the observations with a more physiological perfusion fluid, as a check against the possibility that Locke's solution conditioned the responses.

#### *Amines in defibrinated blood*

Similar tests of vasoconstrictor potency were made with the same amines in defibrinated cat's blood instead of Locke's solution. Three cats were exsanguinated for each experiment, the bloods being defibrinated, pooled, and diluted with an equal volume of physiological salt solution. A hindleg from the last cat bled was perfused immediately with this blood, using the same technique as with Locke's solution. Pressures of 150–200 mm. Hg were necessary to get an adequate rate of flow, suggesting that either the greater viscosity of the blood, or its vaso-



constrictor action, diminished the flow. Table I presents the average ratios obtained.

The catechol and metahydroxy-phenyl derivatives had somewhat greater activities in blood than in Locke's solution, although of the same general order of magnitude. Of the eight compounds in this group, five were not affected by the change of perfusion fluid more than might occur from chance variation, as shown by the standard errors. Of the remaining three, one was the exceptional ethylnorsuprarenin, which should not be considered with the others. The average increase in activity in blood for the entire group was 2.05. This increased response was due probably to better oxygenation of the tissues by the more physiological blood, which permitted better tonus of the blood vessels. Possibly also with a slower rate of flow, there was a longer exposure of the blood vessels to the action of the amines.

The remaining group of agents, comprising the last nine in Table I, were the ortho- and para-hydroxy-phenyl and non-substituted phenylic ring compounds which were relatively inactive in Locke's solution. Of these, benzedrine and diphenylethylamine had such limited vasoconstrictor power that they have been omitted from this comparison. The other seven agents showed good vasoconstrictor potency in blood, entirely comparable with that of the first group, which was in striking contrast to their lack of vasomotor action when used in Locke's fluid. Ortho-oxyephedrine in Locke's fluid caused only vasodilatation, but had one-eleventh the vasoconstrictor action of adrenaline in blood. All the remaining agents were likewise more potent in blood, both the average and median increases in activity for the group being between 200 and 300 times. Phenylethylamine and phenylethanolamine responses were affected less than the other phenyl derivatives by the blood, yet they were rendered eleven and twenty times, respectively, as potent as judged by the mean responses. This is a considerably greater increase in potency than was produced with any of the meta- or dihydroxy-compounds. In view of this, and the chemical relationships, it seemed justified to include them in the group desensitized in Locke's fluid.

Therefore, there was the striking contrast that amines with the dihydroxy- or metahydroxy-phenyl nucleus were relatively little affected by the presence of blood in the perfusion fluid, whereas the other amines were made some hundreds of times more potent by the same condition. Conversely, Locke's solution failed to provide the conditions necessary for responses of these latter agents. This indicated some fundamental difference in the mechanisms of vasoconstrictor action. The remainder of this paper is concerned with this difference.

*Stability of the standard of reference (adrenaline)*

An objection to the suggested difference in actions of the amines might be that it was not the amine response which changed in blood, but rather the response to adrenaline, the standard of reference. Against this objection was the fact that the ratios for all the amines did not shift in the same way, as they would have done if the variation had been in the adrenaline responses. In addition, a direct measure of the absolute adrenaline activity was easily made by comparing the responses to it at

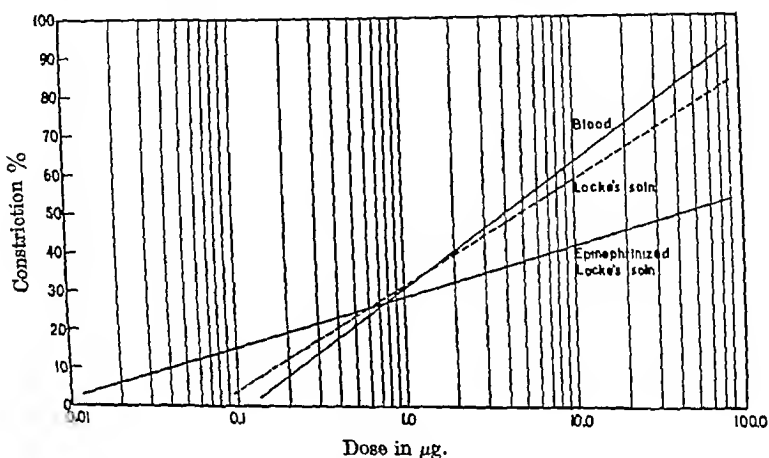


Fig. 1. Average percentage vasoconstrictions produced by the injection of graded doses of adrenaline (epinephrine) into the perfusion system of excised cat's legs when the perfusion fluid was diluted defibrinated blood, normal Locke's fluid, or Locke's fluid with adrenaline (epinephrine) added in concentrations between 1:10,000,000 and 1:2,500,000.

different dosage levels when the two perfusion fluids were used. For this comparison there were available 705 responses to adrenaline in Locke's fluid and 384 in blood. A substantially linear relationship was found between the log-dose and percentage vasoconstrictions, so that direct calculation of the equations of the lines from the grouped data was readily accomplished (Fig. 1).

It is seen that the two dose-response lines were practically identical, both as to slope and location. The deviations between them were considerably less than might have been anticipated from the inherent variability of the individual responses. Over one-half of the results were obtained with doses of from 1 to 2 µg., and in this range the differences between the responses in blood and Locke's were smaller than the

accuracy with which the minute flow was measured. This clearly demonstrated that the response to adrenaline was practically the same in the two fluids, and that any changes observed in the relative activities of the other amines were the result of shifts in their potency rather than to adrenaline.

#### *Vasomotor state in Locke's fluid*

The loss of potency in Locke's fluid might have been conditioned by a lowered initial tonus of the blood vessels, as mirrored in the lowered perfusion pressures required to produce given rates of flow. Evidence on this was obtained by grouping the ratios for the agents according to the tonus of the vessels at the time of injection. However, no correlation between these factors could be demonstrated. More direct evidence on this suggestion is presented farther on, where a direct alteration of the vascular tonus also did not alter the ratios.

#### *Progressive loss of vasomotor activity*

Another possibility considered early was that the loss of response in Locke's fluid was partly a progressive failure in reactivity of the leg vessels which affected some agents before others. This should have been more marked near the end of an experiment than in the beginning. The data were grouped with reference to the total duration of the perfusion before the amine response was elicited from which each ratio was derived. In addition, special perfusions were made with Locke's fluid where the first injection was the amine in question. However, these methods gave no indication that the failure to get reactions in Locke's fluid from some of the agents was a part of a general progressive failure of responsiveness of the leg vessels. Similarly, there was no correlation between the total volume of Locke's fluid which had been perfused through, and the magnitude of the ratios of desensitized agents.

#### *Constant versus pulsatile perfusion pressures*

Another possibility in the different reactions of the amines was that a constant head of pressure was less physiological than the natural intermittent or pulsatile pressure. Accordingly, the driving system for the perfusion was changed to a pump which rhythmically forced the fluid through the leg. Since this produced an almost constant flow with the pressure reflecting the arteriolar resistance, a mercury manometer was used as in ordinary blood-pressure arrangements. The ratios of vasoconstrictor power were calculated as in the previous experiments, using the percentage increases in pressure as measures of constriction. The results obtained are summarized in Table II. Cobefrine,

neosynephrine, tyramine and ephedrine were selected as typical representatives of the various groups, and the determinations were restricted to these.

TABLE II. Vasoconstrictor activity of sympathomimetic amines in terms of ratios to adrenaline when cat's leg vessels were perfused with Locke's fluid under pulsatile pressure

Fluid used	No. of cats	Ratios to adrenaline (mean $\pm$ s.d. (median))			
		Cobefrine	Neosynephrine	Tyramine	Ephedrine
Locke's	6	5.7 $\pm$ 1.9 (3.9)	16.5 $\pm$ 2.7 (14.3)	986 (Only 1 response obtained in 6 legs)	3960 (Only 1 response obtained in 6 legs)
Locke's plus 0.01 % curare	6	45.0 $\pm$ 10.4 (49.5)	188 $\pm$ 56.8 (151)	9000 (Only 1 out of 6 legs responded)	8929 (Only 1 out of 6 legs responded)
Locke's plus 0.003 % sodium ascorbate	5	8.2 $\pm$ 0.61 (8.3)	24.0 $\pm$ 1.6 (22.8)	955 $\pm$ 206 (893)	7644 $\pm$ 6391 (9500) (Only 3 out of 5 legs responded)

It is seen that, in five of the six legs tested with tyramine and ephedrine, no responses could be elicited. Therefore, there was a practically complete lack of activity, as in constant-pressure perfusions with Locke's fluid. With cobefrine and neosynephrine the activity was greater than before in Locke's fluid, although well within the range of expected variability. The use of pulsatile pressures, therefore, did not materially alter the qualitative or quantitative responses of the blood vessels.

#### *Influence of skeletal muscles*

The possibility was considered that the skeletal muscles caused changes in the rate of perfusion, through mechanical or hormonal effects on the blood vessels. To test this possibility, six cats were completely curarized, and, after amputation of the legs, the perfusions were made with pulsatile pressure and Locke's fluid containing 0.01 % curare to maintain the paralysis of the nerves. Completeness of the curarization was proven in each leg by electrical stimulation of the sciatic nerve. Table II summarizes the results obtained with the four representative amines.

It is seen that both tyramine and ephedrine were again practically completely inactive. However, cobefrine and neosynephrine produced good responses, although the calculated ratios to adrenaline indicated a marked drop in relative activity. In comparing the absolute responses with those obtained in Locke's fluid, the adrenaline activity was found to be increased about ten times under curare, as judged by the dose

required to produce a given degree of response. This accounted completely for the apparent decrease in the potency of cobefrine and neosynephrine. The mechanism of the potentiation of adrenaline responses under curare was not studied further.

#### *Ascorbic acid of perfused blood*

With the same pulsatile pressure and Locke's fluid, a test was made of the ascorbic acid (vitamin C) content of the blood as a possible essential factor in the difference in the amine responses. This seemed a possibility worth testing in view of the redox activities of both adrenaline and ascorbic acid. Accordingly, 30 mg. of sodium ascorbate were added to each litre of Locke's fluid in a series of five legs, and the ratios determined as before. The results obtained (Table II) were similar to those of other conditions (Tables I and II), and therefore no indication was obtained of some special influence of this oxidation-reduction agent.

#### *Heparinized plasma perfusions*

It was pointed out above that the better vasomotor response in blood to the amines, especially the ortho- and para-hydroxy and non-substituted phenylic groups, might be associated with better nutrition afforded the blood vessels by the blood over that of Locke's fluid. Whether this was a matter of oxygenation was tested directly by using plasma free of red cells instead of defibrinated blood. Accordingly, the constant-pressure system was used to perfuse a series of legs with plasma obtained by centrifuging the heparinized blood. In Table III it can be seen that the responses to cobefrine, tyramine and ephedrine in plasma were in close agreement with those in defibrinated blood. Neosynephrine was somewhat more active in plasma than under any of the other conditions used, but not so much more, in view of the limited number of legs and variability of the individual results, to establish a specific effect of the plasma. The important information afforded by this series of perfusions was that lack of oxygen-carrying capacity of Locke's fluid was not the factor which interfered with the responses to the tyramine-ephedrine group of amines.

#### *Vasoconstrictor effect of perfused blood*

Shed or defibrinated blood is known to produce vasoconstrictor effects, ascribed to the presence of a "vasotonin" [Bornstein, 1926; Von Euler, 1931, 1932]. This agent has sympathetic-like actions, as shown by its constrictor effects being abolished by ergotamine, like those of adrenaline

TAB. III. Influence of the perfusion fluid on the vasoconstrictor ratios of cobefrine, neosynphrine, tyramine and ephedrine to adrenalline in perfused cat's legs under constant perfusion pressure

	Cobefrine			Neosynphrine			Tyramine			Ephedrine		
	No. of cats	Ratios mean $\pm$ s.d. (median)	Fluid/blood mean (median)	No. of cats	Ratios mean $\pm$ s.d. (median)	Fluid/blood mean (median)	No. of cats	Ratios mean $\pm$ s.d. (median)	Fluid/blood mean (median)	No. of cats	Ratios mean $\pm$ s.d. (median)	Fluid/blood mean (median)
Perfusion fluid												
Dofibrinated blood	10	3.2 $\pm$ 0.37 (2.7)	—	8	11.1 $\pm$ 0.08 (0.7)	—	14	28.0 $\pm$ 5.1 (27.6)	—	8	71.0 $\pm$ 10.5 (68.6)	—
Heparinized plasma	8	3.1 $\pm$ 1.0 (2.3)	—	8	3.0 $\pm$ 0.92 (3.2)	—	8	32.2 $\pm$ 8.6 (20.8)	—	6	105 $\pm$ 30.5 (112)	—
Dofibrinated blood passed through liver	8	0.5 $\pm$ 0.77 (0.3)	—	0	13.1 $\pm$ 2.45 (11.6)	—	0	34.2 $\pm$ 5.52 (32.3)	—	0	80.0 $\pm$ 47.1 (45.8)	—
Combined blood values	35	3.0 $\pm$ 0.43 (3.1)	—	25	0.5 $\pm$ 1.32 (0.0)	—	31	30.2 $\pm$ 3.61 (27.0)	—	23	85.6 $\pm$ 20.4 (60.7)	—
Locke's	10	0.9 $\pm$ 1.31 (3.6)	2.5 (2.8)	13	22.6 $\pm$ 4.31 (22.2)	2.4 (2.5)	14	50.17 $\pm$ 14.70 (33.4)	106 (123)	15	311.00 $\pm$ 90.44 (54.35)	363 (81.5)
Locke's plus 0.5 % gelatin	11	0.3 $\pm$ 1.0 (10.2)	2.4 (3.3)	10	8.8 $\pm$ 1.20 (8.8)	0.03 (0.08)	6	73.0 $\pm$ 106 (63.7)	21.1 (25.4)	11	100.1 $\pm$ 75.8 (101.2)	11.6 (15.6)
Locke's plus 0.05 % CaCl <sub>2</sub>	11	4.2 $\pm$ 1.2 (2.4)	1.1 (0.77)	11	7.3 $\pm$ 2.50 (4.1)	0.77 (0.46)	11	99.6 $\pm$ 55.8 (38.2)	33.0 (44.1)	9	22.28 $\pm$ 120.2 (28.6)	26.0 (4.3)
Locke's calcium free	0	12.7 $\pm$ 2.36 (13.7)	3.3 (4.4)	10	20.2 $\pm$ 4.80 (14.4)	2.1 (1.6)	10	50.2 $\pm$ 81.8 (50.0)	10.6 (18.5)	10	109.1 $\pm$ 54.0 (140.5)	23.3 (22.0)
Locke's plus thyroxin 0.0001 %	5	14.0 $\pm$ 1.50 (13.5)	3.7 (4.4)	5	50.3 $\pm$ 5.02 (52.4)	5.3 (5.8)	5	132.1 $\pm$ 43.3 (102.3)	43.8 (37.0)	5	79.13 $\pm$ 123.5 (75.37)	92.4 (116)
Locke's plus insulin 100 U/litre	5	10.0 $\pm$ 3.80 (0.1)	2.8 (2.6)	5	31.7 $\pm$ 8.18 (33.2)	3.3 (2.7)	5	80.7 $\pm$ 25.5 (77.8)	20.7 (28.8)	5	51.26 $\pm$ 31.80 (13.77)	62.2 (20.6)
Locke's plus sol. posterior pituitary 0.04 %	4	23.8 $\pm$ 5.06 (25.5)	6.1 (8.2)	4	32.0 $\pm$ 4.23 (34.7)	3.4 (3.6)	5	50.5 $\pm$ 27.0 (32.5)	10.4 (12.0)	5	127.4 $\pm$ 0.30 (35.7)	14.0 (5.4)
Locke's plus BaCl <sub>2</sub> 0.02-0.04 %	5	24.2 $\pm$ 3.08 (20.2)	0.2 (0.5)	5	22.2 $\pm$ 2.77 (20.4)	2.3 (2.3)	5	46.1 $\pm$ 61.1 (50.0)	15.2 (18.5)	5	70.8 $\pm$ 91.2 (83.3)	9.3 (12.6)
Combined Locke's values	60	11.8 $\pm$ 1.07 (10.7)	3.0 (3.5)	63	21.2 $\pm$ 2.07 (17.4)	2.2 (1.6)	61	176.1 $\pm$ 41.5 (61.1)	58.4 (23.8)	65	820.8 $\pm$ 244.0 (137.7)	98.0 (20.7)

[Heymans, Bouckaert & Moraes, 1932*a, b*]. This "vasotonin" is partly responsible for the higher perfusion pressures necessary with defibrinated blood. Passage of the blood through the lungs of a Starling heart-lung system, or the liver, or storing the blood in an ice box for several days [Bornstein, 1926; Von Euler, 1931, 1932], causes disappearance of this constrictor effect, presumably owing to destruction of the "vasotonin". It seemed unlikely that the presence of this material in the defibrinated blood conditioned the good responses to the tyramine-ephedrine group of amines, since it is practically absent in heparinized blood [Heymans *et al.* 1932*a, b*], which, in our perfusions, gave responses like those with defibrinated blood. However, a direct test of the possibility was made by perfusing legs with defibrinated blood free of "vasotonin".

For this purpose, a secondary perfusion circuit was arranged whereby the main store of defibrinated blood was passed continuously through the lungs or the liver. Blood was withdrawn at intervals from the reservoirs in this circuit to supply the constant-pressure circuit perfusing the leg. The blood passed through the lungs or liver at least three or four times for each circuit of the leg system, thus offering ample opportunity for the removal of the "vasotonin". The blood was kept oxygenated, when the lungs were used, by rhythmic inflation with an artificial respiration machine. These lung experiments were relatively unsatisfactory because of the development of pulmonary oedema and incomplete removal of the "vasotonin", as indicated by the higher pressures required in the leg system. Therefore, the liver was substituted with better results.

The procedure finally adopted was perfusion of the liver through the portal vein with a head of pressure from a column of blood in a glass cylinder equivalent to the normal portal pressure. The blood was oxygenated by placing in the bottom of the cylinder a small Berkefeld filter candle through which oxygen was blown into the blood. The large surface area of the oxygen bubbles, together with adjustment of the rate of flow of the gas to about that which the blood could absorb, provided complete arterialization without serious foaming. With this system the rates of flow in the leg reflected no appreciable "vasotonin" action. Any failure of the liver to function properly was quickly indicated by arterialized blood emerging from the hepatic vein, and by alterations in the reactivity of the leg vessels. The pressor ratios of typical amines in this "vasotonin-free" blood are summarized in Table III.

It is seen that the activity of the amines was not affected by the absence of "vasotonin", since the ratios were similar to those of other

blood perfusions. Therefore, no basis was demonstrated for the assumption that "vasotonin" might be responsible for the reactivity of the perfused blood vessels to the amines in question.

### *Viscosity of blood and Locke's fluid*

Thus far it is seen that it was the plasma which made it possible for the tyramine-ephedrine group to produce their normal vasoconstriction. The problem was, therefore, narrowed down to determining the property or constituent of plasma essential for the vasoconstriction. An obvious physical difference between plasma and Locke's fluid was the viscosity. By adding 0.5% gelatin to Locke's fluid, a mixture isoviscous with the diluted defibrinated blood at the perfusion temperature was obtained as determined by measurement with an Ostwald viscosimeter. When this mixture was used for the perfusion fluid, the ratios obtained for cobefrine and neosynephrine were similar to those with the various bloods and plain Locke's fluid (Table III). However, with tyramine and ephedrine, the ratios were twenty-four and eleven times, respectively, as high as they were in blood. This indicated a considerably greater activity than that in Locke's fluid alone, but still only a small fraction of that in blood.

### *Effect of calcium*

A chemical difference between defibrinated blood and Locke's fluid is the calcium content, which is diminished in the processes of coagulation and of defibrination. To explore the effects of the range of possible calcium concentrations, two special Locke's fluids were used, one in which the calcium content was increased by five times the normal amount to 0.06%, and one in which calcium was absent. The average ratios (Table III) showed no specific action of calcium on the action of the amines, unless it was some increase in the potency of cobefrine and neosynephrine in the calcium-high fluid. As a matter of fact, there was probably more of an increase in absolute response to these agents than the average ratios indicated, since the adrenaline responses were definitely sensitized by high calcium. However, the tyramine and ephedrine ratios still indicated that there was almost no constrictor potency in these agents regardless of the amount of calcium in the perfusion fluid.

### *Thyroxin and insulin*

Hormones with specific metabolic actions are thyroxin and insulin. In a few experiments, 0.0001% thyroxin, or 100 units of insulin per litre, were added to the Locke's fluid. The values obtained for the activity of



the amines all fell within the range of Locke's fluid, so that there was no point in multiplying negative results by running a larger number of determinations on these.

#### *Pituitary and barium*

Posterior pituitary solution contains a hormone with direct action on peripheral blood vessels, which may be present in the defibrinated blood. Therefore, this was added to Locke's fluid in a concentration of 0.04% of posterior pituitary solution U.S.P., which was equivalent to having 2 mg. of international standard powder in each litre of the perfusion fluid. This was the concentration which produced vasoconstriction comparable to that of the defibrinated blood. However, again the tyramine and ephedrine were highly inactive, and showed only about one-fifteenth of their potency in blood. Clearly, post-pituitary hormone was not the constituent of defibrinated blood necessary for the normal vascular reactions to these amines. A similar degree of vasotonic action, produced by barium chloride in from 0.02 to 0.04% strength, also was ineffective in restoring the activity of these amines to that in blood. Since the vascular tonus was high with defibrinated blood, and low with liver-perfused blood, and it was high with pituitary or barium containing Locke's fluid and low with all the other Locke's fluids, it can be seen that the ratios of the tyramine and ephedrine did not follow the level of vascular tonus. Therefore, this factor can be ruled out as the crucial difference responsible for the loss of vasomotor action in Locke's fluid.

#### *Adrenaline added to Locke's fluid*

There remains another hormone with vascular actions, namely adrenaline. This agent plays a role in the responses of blood vessels through humoral transmission of the sympathetic nerve stimulation [Cannon & Rosenblueth, 1937]. Adrenaline injections were alternated with those of the other amines in all the experiments of this study, but it was washed out of the perfusion system to a concentration below that producing a decreased flow before each subsequent injection was made. Since the Locke's fluid, but not necessarily the blood, contained no adrenaline, the amines which were injected during Locke's perfusions acted on blood vessels which were probably washed nearly free of natural adrenaline.

In order to see if the presence of the adrenaline of the blood conditioned these responses, this hormone was added to the Locke's perfusion fluid in various concentrations, and the activity ratios redetermined (Table IV). With cobefrine and neosynephrine, the ratios fluctuated

TABLE IV. Effect of adding increasing concentrations of adrenaline to Locke's perfusion fluid on the ratios of activity for cobefrine, neosynephrine, tyramine and ephedrine. Each of the ratios in this table represents the mean of an average of seven experiments

Adrenaline concentration added	Ratios to adrenaline (mean $\pm$ s.d. (median))			
	Cobefrine	Neosynephrine	Tyramine	Ephedrine
None	9.9 $\pm$ 1.31 (8.6)	22.6 $\pm$ 4.3 (22.2)	5017 $\pm$ 1470 (3334)	31100 $\pm$ 9044 (5435)
1 : 10 million	37.7 $\pm$ 12.7 (40.0)	61.5 $\pm$ 29.9 (56.4)	399 $\pm$ 1163 (313)	619 $\pm$ 269 (377)
1 : 5 million	15.2 $\pm$ 1.72 (15.8)	40.7 $\pm$ 16.2 (28.5)	134 $\pm$ 78.7 (41.0)	190 $\pm$ 94.4 (117)
1 : 2.5 million	11.7 $\pm$ 4.95 (23.5)	29.4 $\pm$ 13.6 (17.5)	39.3 $\pm$ 15.6 (19.1)	30.9 $\pm$ 7.07 (27.8)

within the ranges of those where no adrenaline was added to the perfusion fluid. However, with tyramine and ephedrine, quite a different result was obtained. These two agents were practically completely inactive in plain Locke's fluid, as shown by the ratios in values of thousands. When only a 1 : 10,000,000 concentration of adrenaline was added, the activities of these agents increased many fold, i.e. to 399 and 619, respectively. With 1 : 5,000,000 adrenaline in the Locke's fluid, the ratios dropped further, i.e. to 134 and 190; and with 1 : 2,500,000 adrenaline, they fell to 39 and 31, values as low as, or lower than, those with blood. The concentration of 1 : 2,500,000 adrenaline produced degrees of vasoconstriction comparable to that from defibrinated blood, so that higher concentrations were not tested.

According to these results, adding adrenaline to Locke's fluid resulted in degrees of activity of otherwise inactive amines, entirely comparable with those obtained with them in defibrinated blood. However, the low ratios conceivably might have resulted from a shift in the responses to the injected adrenaline used to establish the ratios of activity. To check this possibility the responses to the adrenaline injections in adrenalized Locke's fluid were tabulated according to doses and the calculated curve drawn as before (Fig. 1). It can be seen that under these conditions the responses increased less rapidly with increasing doses than did those in blood or Locke's fluid. This may have been due to the change in concentration of adrenaline in the tissues being less after the injection of a given dose, when there was already considerable adrenaline present from the perfusion fluid, than when an adrenaline-free fluid was used. The corollary of such a hypothesis might be that the activating substance in blood was not adrenaline, since, otherwise, the responses to adrenaline in blood should have followed those of adrenalized Locke's fluid. There

are alternative possibilities, such as the differences caused by the physical nature of the two fluids, etc., but further speculation on this appears unprofitable here.

Comparison of the curves in Fig. 1 shows that the deviations in the magnitude of responses to adrenaline in the adrenalinized Locke's fluid from those of blood or plain Locke's fluid were not great enough to account for any significant part of the increased activity in the former fluid. The median dose of adrenaline injected in adrenalinized Locke's fluid was  $1.0\text{ }\mu\text{g.}$ , and three-fourths of all the injections fell in the range of  $0.4\text{--}5.0\text{ }\mu\text{g.}$  At the lower end of this range, the shift in response was only equivalent to an upward shift in the adrenaline dose to  $0.5\text{ }\mu\text{g.}$ , and at the upper end, to a downward shift to  $1.7\text{ }\mu\text{g.}$  Therefore, at the most, the order of magnitude of the changes in the ratios of Table IV, introduced by the shift in the adrenaline response, could not be more than three times, and on the average must have been almost nil. Hence, they could not account for the great increase in activity in the adrenalinized perfusion fluid.

#### DISCUSSION

The role of adrenaline in the vascular responses of this study will be recognized as a phenomenon first described by Schaumann for ephedrine in 1928; and by Burn [1930], Burn & Tainter [1931] and others subsequently for tyramine and ephedrine. In his first paper, Burn [1930] reported a tyramine to adrenaline activity ratio of approximately 2000 when the leg was perfused with Ringer's solution. This value dropped to about 50 on the addition of adrenaline to the perfusion fluid. Both values are in line with those obtained by us. In agreement with the present results, Burn also observed [1932] that the vasotonic effect of adrenaline was not the crucial factor, as shown by comparing the effects of increased vascular tonus produced by post-pituitary extract.

The results of this study, therefore, confirm and extend the earlier results on tyramine and ephedrine and establish the order of magnitude of the changes induced by the adrenaline content of the perfusion fluid. Most of the earlier descriptions of the phenomenon were made from comparisons of blood with adrenalinized blood, where the normal adrenaline content operated to make the differences less striking. That shed blood may contain important amounts of adrenaline, which is retained for days, is clearly established by the reports of Bain, Gaunt & Suffolk [1936, 1937]. Our work adds paredrine, *o*-oxyephedrine, propadrine and possibly phenylethylamine and phenylethanolamine to tyramine and ephedrine, whose vasomotor actions were recognized as depending on

adrenaline being available to the peripheral tissues. Our work also establishes that arterenol, 3-4-dioxyephedrine, cobefrine, ethynor-suprarenin, epinine, laevometaoxyephedrine, neosynephrine and meta-oxynorephedrine do not depend on adrenaline being in the perfusion fluid, in order to produce their characteristic vasomotor responses, since the differences in their activities are no greater than might be expected from the non-specific effects of defibrinated blood. It is by no means certain that there is a sharp qualitative difference between these two groups, since phenylethylamine and phenylethanolamine may be intermediate in possibly graded degrees of dependence on the adrenaline supply.

An hypothesis to explain the results obtained can be formulated along the same lines as the theories of Burn [1932], Bülbring & Burn [1938] and Gaddum and Kwiatkowski [1938]. That is to say, those compounds not dependent on the adrenaline supply produce stimulation of the most peripheral sympathetic mechanisms in a way comparable qualitatively but not quantitatively to that of adrenaline liberated from the nerve endings. The sole common chemical characteristic of these agents, which may almost be expected to break down when a more extended series has been studied, is that they have a hydroxyl group in the meta-position of the phenyl ring. The remaining amines act by blocking the ferment in the myoneural junction, which destroys the adrenaline constantly being supplied by the blood stream and nerve endings. There results a localized increase in adrenaline, which causes stimulation, presumably comparable to that of sympathetic nerve stimulation or of adrenaline injection. That such a ferment exists, in analogy to the choline-esterase for the para-sympathetics, is being generally assumed. It is not certain that it is identical with the amine-oxidase of Blaschko, Richter & Schlossmann [1937] until further studies have been made to determine if the inhibition of this particular ferment by the present amines correlates with their hypothetical mechanisms of action. There are other ferments in tissues which destroy adrenaline readily [Blaschko & Schlossmann, 1938], which may play the dominant role in its inactivation, and whose actions in the presence of these other amines remain to be explored.

The principal virtues of the above hypothesis are that it explains various apparently contradictory actions of these amines and it can be readily subjected to experimental verification by application of Shaw's [1938] test to perfusates, as in work on ephedrine by Gaddum & Kwiatkowski [1938]. However, it appears unlikely that amine-oxidase inhibition can explain all the actions of these amines, even in the low-dosage

ranges, since augmentor effects resembling direct muscular stimulation can be demonstrated under selected conditions in organs where injected adrenaline or sympathetic stimulation cause only inhibition. There are other discrepancies, which can be more advantageously considered in connexion with other experimental data.

In Table V are summarized the pressor activities of these amines in intact animals, previously reported from this laboratory, for comparison with the activity demonstrated on peripheral vessels in this study. It is

TABLE V. Comparison of activity of various amines on blood pressure and blood vessels with the dependence of the responses on the presence of adrenaline and the effects of previous cocainization *in vivo*

Amines	Relative pressor activity		Change in activity in legs due to adrenaline	Effect of cocaine <i>in vivo</i>
	Cat's legs perfused with blood	<i>In vivo</i>		
Dihydroxy- and meta-hydroxy compounds:				
Adrenaline	1	1	—	S
Arterenol	1.12	0.9	1.7	S
3-4-Dioxyephedrine	31.4	91.1	0.7	S
Cobefrine	3.2	2.9	3.1	S
Epinine	7.2	7.6	0.85	S
1- <i>m</i> -Oxyephedrine	148	107.7	2.0	—
Neosynephrine	11.1	3.3	2.0	S
<i>m</i> -Oxynorephedrine	48.2	10.7	4.0	Unaltered
Ortho- and para-hydroxy and unsubstituted phenyl compounds:				
Phenylethylamine	6.1	112	10.7	D
Phenylethanolamine	3.8	112	19.8	D
Paredrine	33.4	210	214	—
Tyramine	28.9	118	174	D
<i>o</i> -Oxyephedrine	11.2	410 (?)	∞	—
Ephedrine	71.6	280	434	D
Benzedrine	Mainly dilator	347	—	D
Propadrine	36.5	67.5	892	D

<sup>1</sup> In this column, S=sensitized and D=desensitized.

seen that, generally, though not invariably, the catechol and metahydroxyphenyl compounds (upper half of the table) showed about the same activity on the legs perfused with blood as they did in intact animals, that addition of adrenaline to the Locke's fluid altered their activity relatively little, and that their actions were increased by cocaine. By contrast, other hydroxyphenyl and non-substituted phenylic compounds (lower half of the table) were much less active in intact animals than in blood-perfused legs. Their vasoconstrictor power was largely dependent on adrenaline in the perfusion fluid, and their action is diminished or abolished by cocaine, when the cocainization is carried out by our techniques. Therefore, these groupings seem to indicate a relationship between the "cocaine paradox" phenomenon, the need for adrenaline

in peripheral vascular responses, and the comparatively low degrees of activity in the intact animal. Until the contradictory effects of cocaine are better understood, there seems little chance that the possible fundamental significance of these correlations can be definitely established. However, it should be pointed out that if cocaine could be demonstrated to act by the same mechanism as the ephedrine one suggested above, the "cocaine paradox" would be reasonably explained. Experiments to test this will be reported shortly.

#### SUMMARY AND CONCLUSIONS

1. Cat's hindlegs were perfused with defibrinated blood or Locke's fluid, and the vasoconstrictor power of a series of representative sympathomimetic amines was determined.

2. When defibrinated blood was the perfusion fluid, the vasoconstrictor activities of the compounds having a catechol nucleus or a hydroxyl group in the meta-position in the benzene ring were of the same general order as their activities *in vivo*, whereas those with an ortho- or para-hydroxyl or unsubstituted phenyl ring tended to be considerably more active on these perfused vessels than they were in intact animals.

3. When Locke's fluid was the perfusion fluid, the first group of compounds retained their vasoconstrictor power almost as well as in blood, whereas the second group was greatly diminished in potency, or almost completely inactive.

4. The responses to the former group of amines have been previously shown to be sensitized, and the latter group to be desensitized, by cocainization in the intact animal according to our technique.

5. Comparison of the effects of four amines representative of these two groups, namely, cobefrine, neosynephrine, tyramine and ephedrine, in defibrinated blood, defibrinated blood freed of "vasotonin" by passage through the liver, or heparinized plasma, with those of the same amines in Locke's fluid alone, or modified by the addition of gelatin, thyroxin, insulin, posterior pituitary solution or barium, or by changes in calcium content, did not reveal a cause for the differences between the two groups.

6. Use of pulsatile pressures, instead of steady perfusion pressures, and normal Locke's fluid or Locke's fluid containing ascorbic acid or curare, also did not significantly affect the differences observed.

7. However, when adrenaline was added to Locke's fluid, the responses to tyramine and ephedrine increased in proportion to the adrenaline concentration of the perfusion fluid. With a 1 : 2,500,000 concentration

of adrenaline in the Locke's fluid, the responses to these amines were comparable to those in defibrinated blood.

8. These results are consistent with the hypothesis that the tyramine-ephedrine group of amines produce at least part of their vasoconstrictor action by blocking the local tissue mechanisms for inactivating adrenaline, thereby permitting increased local concentrations of this hormone, liberated from the sympathetic endings, or derived from the blood stream. The other group of amines appear to act independently of this mechanism, perhaps functioning qualitatively in the same way as adrenaline itself.

9. The fundamental significance of the apparent correlation of the dependence of the vasomotor activities on the presence of adrenaline, their relative effectiveness, or lack of it, in the intact animal as compared with the perfused legs, and their behaviour after cocaineization, appear to merit more extended study.

The following firms or individuals supplied various amines gratis for this study: I G Farbenindustrie Aktiengesellschaft-Hoechst, and the Winthrop Chemical Co., arterenol, 3-4-dioxyephedrine, cobefrine, 1-*m*-oxyephedrine, *m*-oxynorephedrine, *o*-oxyephedrine, Burroughs-Wellcome Co., ephedrine; Frederick Stearns and Co., neosynephrine, Dr Gordon Alles, phenylethanolamine, Smith, Kline and French, pargoline and benzedrine, Sharpe and Dohme, Inc., propadrine; Dr A. Novelli of the Faculty of Medicine, Buenos Aires, diphenylethylamine (1-3 diphenyl 2-amino-propane). The remaining drugs were obtained from the usual commercial sources.

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## THE SUBSTANCE CAUSING RENAL HYPERTENSION

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GOLDBLATT, LYNCH, HANZAL & SUMMERVILLE [1934] have shown that chronic arterial hypertension can be produced by permanent compression of the renal artery. If the ischaemic kidney is extirpated or the arterial compression discontinued, the blood pressure returns to the normal level [Goldblatt *et al.* 1934; Dicker, 1937; Houssay & Fasciolo, 1937*a*; Goldblatt, 1937; etc.].

Houssay & Fasciolo [1937*b*] grafted the ischaemic kidneys of dogs with chronic hypertension into the neck of normal or nephrectomized dogs. An immediate increase in blood pressure of 30-70 mm. Hg was observed, whereas no appreciable change was produced by grafting normal kidneys. Bouckaert, Grimson & Heymans [1939] have confirmed these findings which clearly demonstrate that ischaemic kidneys produce a pressor substance.

With a different technique Houssay & Taquini [1938*a*] have studied the appearance of the active principle. Venous blood of ischaemic or normal kidneys was collected in a "cava pocket", diluted with citrate and Ringer solution and then tested on the Lâwen-Trendelenburg preparation. The citrated plasma of the venous blood of ischaemic kidneys was found to produce vasoconstriction [Fasciolo, Houssay & Taquini, 1938].

Pressor or vasoconstrictor properties of the renal venous blood appear not only in chronic ischaemia of the kidney, but can also be detected a few minutes after the compression of the artery of a normal kidney [Enger, Linder & Sarre, 1938; Verney & Vogt, 1938*a*; Grimson, 1939; Braun-Menendez & Fasciolo, 1939]. We have repeated and confirmed some of the experiments on the pressor and vasoconstrictor properties of the blood from ischaemic kidneys and studied some of the properties of the active substance of this blood. This substance was found to be different



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Pressor or vasoconstrictor properties of the renal venous blood appear not only in chronic ischaemia of the kidney, but can also be detected a few minutes after the compression of the artery of a normal kidney [Enger, Linder & Sarre, 1938; Verney & Vogt, 1938*a*; Grimson, 1939; Braun-Menendez & Fasciolo, 1939]. We have repeated and confirmed some of the experiments on the pressor and vasoconstrictor properties of the blood from ischaemic kidneys and studied some of the properties of the active substance of this blood. This substance was found to be different

from the pressor kidney protein "renin" which has been studied by Tigerstedt [1897], Hessel [1938], Pickering & Prinzmetal [1938], Helmer & Page [1939], Swingle, Taylor, Collings & Ways [1939], etc. But it was found that by the action of renin on blood proteins a substance is formed which has the same chemical and physiological properties as the pressor substance from the venous blood of ischaemic kidneys. This substance was named "hypertensin" [Braun-Menendez, Fasciolo, Leloir & Muñoz, 1939; Muñoz, Braun-Menendez, Fasciolo & Leloir, 1939].

#### A. PROPERTIES OF THE VENOUS BLOOD OF KIDNEYS WITH ACUTE ISCHAEMIA

##### (1) *Pressor action*

(a) *Experiments with a kidney grafted into the neck of a normal dog.* When a normal kidney was grafted into the neck of a normal or nephrectomized dog, no changes of blood pressure occurred. But if the renal artery of the graft was partially compressed, an increase in blood pressure was produced (about 30 mm. Hg in 5-6 min.). With a previous ischaemia of 60-90 min. the effect of the graft was greater.

In order to study the pressor properties of the venous blood of these ischaemic kidneys, experiments were carried out as follows. The renal artery of a normal dog under chloralose anaesthesia was compressed. After 40-80 min. the kidney was removed and the renal artery connected to the carotid of a second dog. This second dog was previously treated with chloralose and an anticoagulant (chlorazol fast pink, 0.08 g./kg. body weight). The venous blood of the grafted kidney was then collected. The first 20 c.c. were discarded and the next portion (100 c.c.) flowing at a rate of 10-15 c.c./min. was kept for the test. Blood from the jugular vein or from the vein of normal kidney was used as control. When the blood pressure of the second dog was quite steady, the blood samples were slowly (1-2 min.) injected into the jugular vein.

Experiments carried out in this manner showed that the blood of the ischaemic kidney always produced greater increases in blood pressure than the control blood (Table I).

TABLE I. The action of venous blood of kidneys grafted into the neck

	Rise in blood pressure (mm. Hg) by injection of 20 c.c. of plasma							Average
	32	28	17	16	70	23	16	
Venous blood of ischaemic kidney								28.8
Control blood	16	18	10	10	20	10	10	13.2

The compression of other arteries (femoral, duodenal) did not confer pressor properties on the corresponding venous blood.

(b) *Experiments with Starling's heart-lung preparation.* The technique was as follows: The heart-lung preparation was prepared in the classical manner using a reservoir of 1500 c.c. Defibrinated blood was circulated for 90–120 min., a time which, as we have found, is sufficient for the disappearance of its vasoconstrictor action [Braun-Menendez & Fasciolo, 1939]. The left kidney of a large dog (15–18 kg.) was then extirpated and

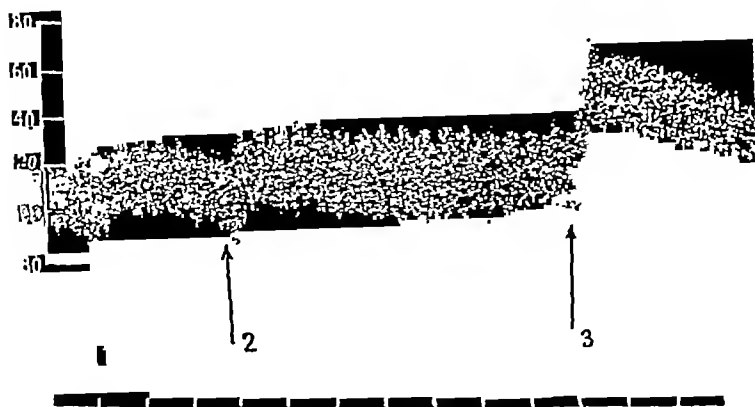


Fig. 1. The pressor action of blood from ischaemic kidney. Dog, 14 kg., chloralosed and nephrectomized. Femoral blood pressure (mm. Hg.). Time in minutes. 1, intravenous injection of 15 c.c. serum of circuit's blood; 2, 15 c.c. serum of circuit's blood after 10 min. asphyxia of the heart-lung preparation; 3, 15 c.c. serum of venous blood of perfused ischaemic kidney.

rapidly grafted to the arterial side of the heart-lung preparation following Verney & Starling's [1922] technique. The venous inflow and arterial pressure of the preparation were then regulated so that the flow through the kidney was from 100 to 250 c.c./min. according to its size. These conditions were maintained for 5 or 10 min., the kidney's venous blood being returned to the circuit. After this time, samples of venous blood were collected and then ischaemia was produced by decreasing the venous inflow to the heart and the arterial pressure, or, if necessary, by compressing the tube leading to the renal artery. The renal blood flow was reduced to 10–20 % of the original value. The samples of venous blood of ischaemic kidney and in many cases control samples of blood from the

circuit were then collected, the blood was centrifuged and the serum, 10-20 c.c., was injected intravenously into chloralosed dogs which 1 or 2 hr. before had been injected with 5-10 c.c. of blood from the circuit. In this manner the shock that the injection of the serum samples would otherwise produce was avoided.

The results are shown in Table II.

TABLE II. The action of venous blood of kidneys perfused by the heart-lung preparation

	Rise in blood pressure (mm. Hg) per 20 c.c. serum injected																			
	28	31	17	42	70	32	30	40	13	25	30	22	40	12	18	37	53	53	53	30
is blood of isemic kidney																				
is blood of ial kidney	8	10	20	.	16	12	.	.	13	.	0	.	.	.	.	.	.	27	.	.
from circuit	.	.	.	12	14	12	13	26	13	5	0	10	20	0	20	22	30	.	30	10

The pressor action of the serum of ischaemic kidneys was rather prolonged (Fig. 1) and was not greatly modified by previous section of the vagi, denervation of the carotid sinus, adrenalectomy or injection of atropine (1 mg./kg.), cocaine (10 mg./kg.), or Fournau 933 (10 mg./kg.).

## (2) Vasoconstrictor action

(a) *Experiments with kidneys grafted into the neck of normal dogs.* Following exactly Houssay & Taquini's [1938 a, b], procedure with the L wen-Trendelenburg preparation, it was found that the citrated plasma of the kidney's venous blood did not produce a vasoconstriction when the flow was normal. But after a few minutes of arterial constriction there was a clear effect which disappeared after the constriction was discontinued.

However, this procedure is not quite free from errors, because by coagulation the blood acquires a strong vasoconstrictor action. The blood of ischaemic kidney is collected more slowly and therefore is more liable to coagulate. Even normal samples of blood collected in oxalate or citrate solution may produce vasoconstriction, although coagulation is hardly detectable. It was in order to eliminate this source of error that we used Starling's heart-lung preparation as a perfusion system for the kidney.

(b) *Experiments with the heart-lung preparation.* If defibrinated blood is circulated for 30-60 min. through Starling's heart-lung preparation its vasoconstrictor action on the L wen-Trendelenburg preparation disappears [Braun-Menendez & Fasciolo, 1939].

The samples of serum were mixed with 7 vol. of Ringer solution and perfused through the L wen-Trendelenburg preparation. The serum obtained under the described conditions, from the blood of normal kidneys

with normal blood flow, had no action, whereas that from the same kidney after 10-20 min. ischaemia produced an intense vasoconstriction.

These results agree with those of Houssay & Taquini [1938 *a, b*]. Verney & Vogt [1938*b*] observed a fall in blood-flow through the gut when a normal isolated kidney perfused by one heart-lung circuit was switched to another by which an excised loop of gut was being perfused.

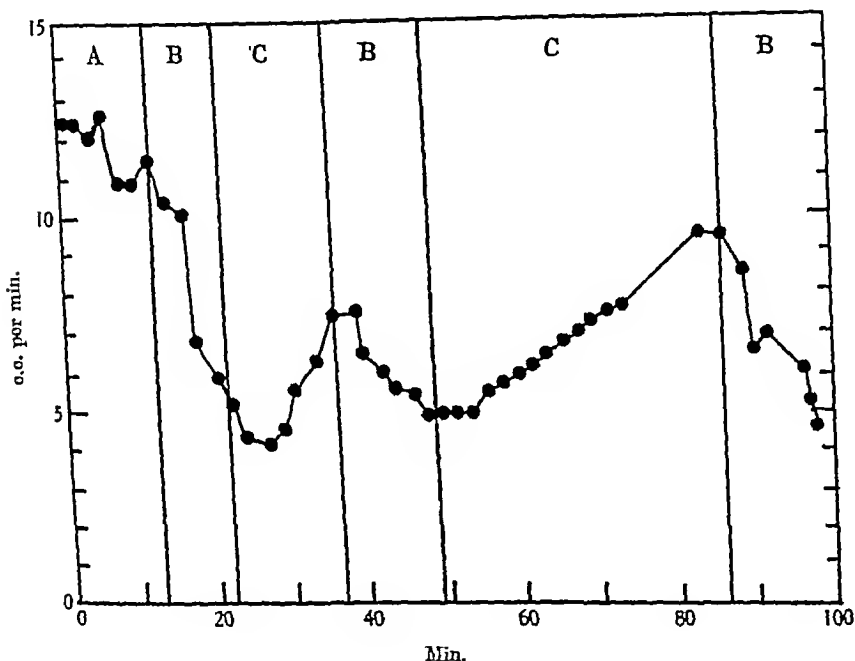


Fig. 2. The vasoconstrictor action of blood from the ischaemic kidney. Perfusion of the denervated leg of a dog with defibrinated blood: A, blood from the heart-lung circuit after functioning 1 hr.; B, venous blood of ischaemic kidney; C, venous blood of kidney with normal flow.

In three experiments the vasoconstrictor (Läwen-Trendelenburg preparation) and the pressor action of different samples of blood were compared. It was found that both actions were parallel.

A few perfusions of the denervated dog's leg with defibrinated blood disclosed a marked vasoconstrictor action of the venous blood of ischaemic kidney. The decrease in venous flow was taken as a measure of vasoconstrictor action (Fig. 2).

(3) *Other properties*

The usual plethysmographic methods did not reveal contraction of the leg or kidney or any alterations in the motility of the intestine. But it was found that the injection of blood from the ischaemic kidney produced a net decrease in the volume of the denervated spleen.

No effect was observed on the denervated dog's heart (heart-lung preparation).

## B. THE PRESSOR SUBSTANCE FROM THE BLOOD OF THE ISCHAEMIC KIDNEY

(1) *Preparation of extracts*

Blood with pressor properties was obtained, as previously described, and centrifuged. The plasma or serum was then precipitated with 3 vol. of acetone. The filtrate was freed from acetone by distillation under reduced pressure and thoroughly extracted with ether, after making alkaline to phenolphthalein and then acid to congo red. After neutralization and, if necessary, filtration, the solution was ready for injection.

(2) *Chemical and physiological properties*

Extracts of nineteen different samples of blood of ischaemic kidneys were injected intravenously into nineteen chloralosed dogs. In each case a control extract was prepared with blood from the carotid artery or the jugular vein or from the renal vein of non-ischaemic kidneys.

The extracts from ischaemic kidney blood always produced a blood-pressure increase lasting 3-4 min. (Fig. 3), whereas the controls only produced it in four samples out of nineteen (Table III).

TABLE III. Rise in blood pressure (mm. Hg) after injection of acetone extracts of 50 c.c. serum

	Ischaemic kidneys grafted into the neck of normal dogs							Ischaemic kidneys perfused by a heart-lung preparation											
Ischaemic blood of	35	60	40	54	38	62	16	20	46	22	20	30	20	20	25	30	35	15	
Ischaemic kidneys																			
Control blood	.	20	0	40	10	0	0	6	0	0	.	0	0	0	.	0	.	.	.

As large amounts of venous blood of ischaemic kidneys are difficult to obtain, only a few of the properties of the pressor substance were studied. These properties were found to be the same as those of the substance formed *in vitro* which are described later.

The pressor effect was not modified if the dogs were previously treated with Fourneau 933 (Fig. 4). After the substance was subjected to *N HCl* at 100° C. for 2 hr., no great decrease in activity occurred. The substance

is insoluble in ether or amyl alcohol, soluble in water, 96 % alcohol and glacial acetic acid and may be kept unaltered for long periods in solution or dried.

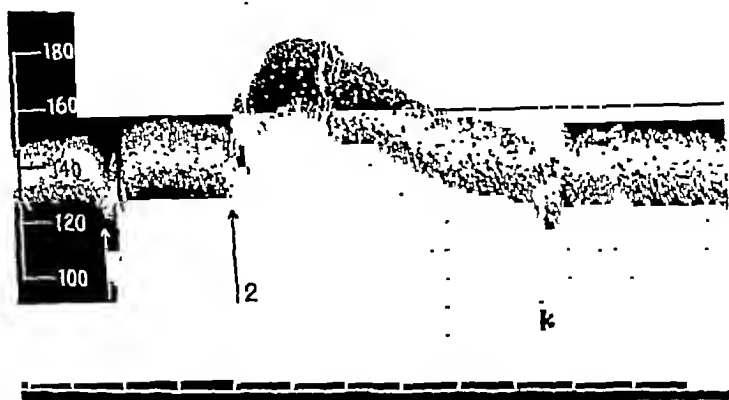


Fig. 3. The pressor action of extracts from the blood of ischaemic kidneys. Dog, 6 kg., chloralosed. Double vagotomy and artificial respiration. Femoral pressure in mm. Hg. Time in minutes. Intravenous injection of acetone extracts of 50 c.c. serum: 1, of circuit's blood 1 hr. after completion of the heart-lung preparation; 2, of venous blood of perfused ischaemic kidney; 3, of circuit's blood 3 hr. after completion of preparation.



Fig. 4. Effect of Fourneau 933 on the pressor action of blood extracts. Dog, 8 kg., chloralosed and nephrectomized. Double vagotomy and artificial respiration. Femoral pressure in mm. Hg. Time in minutes. Between 2 and 3, intravenous injection of 80 mg. of Fourneau 933. 1, intravenous injection of acetone extract of 50 c.c. serum from venous blood of ischaemic kidney; 2, the same after 10 min. at 100° C. at pH 2; 3, the same; 4, 10  $\mu$ g. adrenaline.

The acetone precipitate of serum, after being washed with acetone and ether, dried and dissolved in 1 % NaCl solution, was also tested. It generally produced a decrease in blood pressure.



C. THE PRESSOR SUBSTANCE FORMED *IN VITRO*

If blood serum is incubated at 37° C. with renin, a pressor substance is formed which is apparently the same as that which was found in the blood of ischaemic kidneys.

A typical experiment is shown in Fig. 5.



Fig. 5. Formation of hypertensin *in vitro*. Dog, 12 kg., chloralosed. Both vagi cut. Artificial respiration. Femoral blood pressure in mm. Hg. Time in minutes. 1, 20 c.c. of horse serum and 0.5 c.c. renin solution incubated separately and mixed during precipitation with alcohol; 2, the same incubated together 15 min. at 37° C. and then precipitated; 3, same as 1 but with bovine serum; 4, same as 2 but with bovine serum.

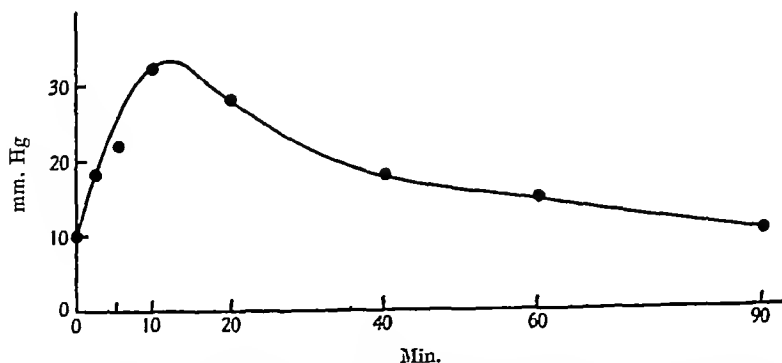


Fig. 6. Blood-pressure increases produced by acetone extracts from 10 c.c. of blood globulins (=20 c.c. serum) incubated at 37° C. with 0.25 c.c. renin (=1 g. fresh kidney) for various times.

The same results were obtained with bovine, horse and dog serum. The necessary component was found to be a blood protein (pseudoglobulin) which is precipitated by half-saturation with ammonium

sulphate and not by dialysis. Haemoglobin, casein, milk serum, and egg or blood albumin were tested with negative results.

The time of reaction with the renin and its concentration have a great importance on the amount of pressor substance obtained. The effect of time is shown in Fig. 6. The formation is rapid and there is afterwards a gradual disappearance of the pressor principle. If the amount of renin is increased the amount of pressor substance is not greater but smaller, whereas with a constant amount of renin more substance is formed if the amount of blood globulins be increased.

By incubation of blood globulins with renin which had been heated for 5 min. at 100° C. no active extracts were obtained. Many experiments were carried out using pepsin, trypsin or papain or extracts of liver and spleen instead of renin. With none of them was a clear formation of pressor substance obtained.

The pH optimum is difficult to ascertain. By taking several points in the time curve (Fig. 6) with small amounts of renin, it was found that the formation of hypertensin is faster at a pH around 7.

The amount of hypertensin obtained from 20 c.c. serum under the best conditions caused a blood-pressure increase of 20-30 mm. Hg and was considered as one unit.

#### (1) *Larger scale preparation*

*Blood globulins.* Bovine blood (40 l.) was collected in the slaughter-house, 0.2 % potassium oxalate being added. After centrifugation, the plasma (about 20 l.) was precipitated with ammonium sulphate (400 g./l.), and filtered first through fluted paper and then through Büchner funnels in order to free it as much as possible from the residual liquid. The cakes were then dialysed overnight in cellophane bags against running water. The globulins (about 10 l.) were then filtered through muslin.

*Renin.* Pig kidneys were finely minced, 3 vol. of 2 % NaCl and 5 % toluol were added and left overnight at room temperature. The toluol and fats were then skimmed off and the rest filtered through cloth. Acetic acid (5 N) was added to the filtrate until it gave a green colour with brom-cresol-green. After centrifuging the yellow supernatant liquid was precipitated with 380 g./l. ammonium sulphate. After filtering through Büchner funnels the cake was dialysed overnight. The liquid was then filtered: 1 c.c. corresponded to 3-4 g. fresh kidney and when injected intravenously into chloralosed dogs gave a rise in blood pressure of about 50 mm. Hg.

*Hypertensin.* The amount of renin which is to be added depends on the activity of the preparation and was ascertained by small scale trials. Generally it was about 1%. The renin was added to the blood globulins previously heated to 37° C. After 15 min. the liquid was poured into 3 vol. of boiling alcohol (96%). When the mixture had boiled for several minutes, it was cooled and filtered *in vacuo*. The filtrate was evaporated to a small volume under reduced pressure. The water solution was then thoroughly extracted with ether and filtered. Three volumes of ethyl alcohol (96%) were added and the solution left overnight at 4° C. After filtration, the solution was evaporated under reduced pressure. The turbid aqueous solution contains about 2000 hypertensin units with a dry wt. of about 15 mg./unit.

### (2) Chemical properties

The substance is very soluble in water and glacial acetic acid, soluble in 96% alcohol and insoluble in ethyl ether, chloroform and amyl alcohol.

It can be salted out from aqueous solutions by saturating with ammonium sulphate. It dialyses through cellophane and can be precipitated with phosphotungstic acid. In *N* HCl at 100° C. the activity is completely lost after 3 hr. In 0.15 *N* NaOH at 100° C. activity disappears after 10 min. Benzoylation by the Schotten-Baumann method inactivates hypertensin.

A further purification can be obtained by extracting dry preparations with glacial acetic acid and precipitating with ether. This precipitate contains 1 unit in 3 mg.

Salts of heavy metal were not found useful in the purification.

### (3) The destruction of hypertensin

Hypertensin is a stable substance, but it is rapidly destroyed by tissue extracts. As shown in Fig. 6 hypertensin disappears after a certain time when the mixture of renin and blood globulins is incubated. If one unit of hypertensin is incubated for 15 min. at 37° C. with 1 c.c. of renin and afterwards heated for 5 min. at 100° C. to destroy the renin, no pressor effect is obtained on injection. If renin is boiled before the incubation it has no effect, whereas neither cyanide *M*/200 nor anaerobic incubation inhibits the destruction.

Extracts of liver and spleen, and commercial trypsin, pepsin and papain solutions were also found to destroy hypertensin.

Blood serum also possesses this action: 2 c.c. of dog's, horse's or bovine serum inactivate hypertensin completely in 40-60 min. at 37° C., but not

at 4° C. The active fraction of blood was found to be precipitated by half-saturation with ammonium sulphate, but not by dialysis.

Before injecting the hypertensin thus treated, proteins were removed by precipitation with three volumes of alcohol (96 %) and the latter was evaporated under reduced pressure.

#### (4) Pharmacological properties

Hypertensin, prepared as previously described, was used, the aqueous solution being previously neutralized and filtered. One unit was considered as the amount which gave an increase of 20–30 mm. Hg in blood pressure. The responses of different dogs were somewhat variable.

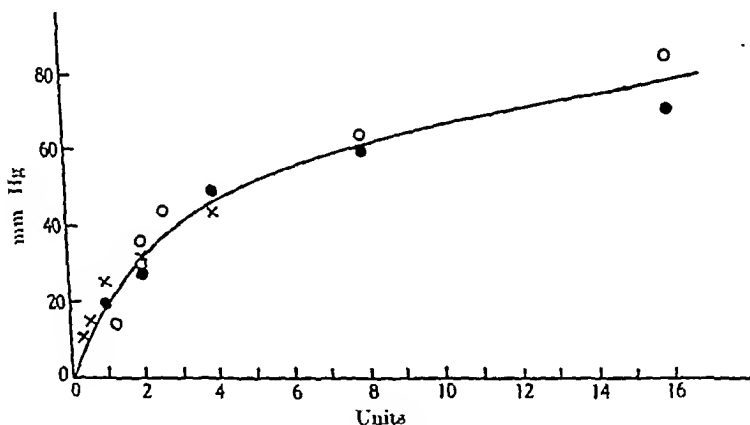


Fig. 7. Increases in blood pressure produced by different amounts of hypertensin.

*Response to different doses.* The increase in blood pressure, which lasts about 3–4 min., is proportional to the amount injected, as shown in Fig. 7.

*Injection into different blood vessels.* Pressor effects were obtained when hypertensin was injected into veins (jugular or saphenous) or into arteries (aorta or carotid). The latent period depends on the site of injection, for the injection into the aorta it was 6 sec.; into the jugular vein, 15 sec.; saphenous vein, 18 sec.; carotid artery, 35 sec. These results suggest that the hypertension is due to a vasoconstriction in the largest vascular beds (the splanchnic area and the limbs).

*Action on different organs.* By the use of plethysmographs it was found that one unit of hypertensin decreases the volume of the spleen and of the kidney. The heart rate was not modified in vagotomized dogs. A small and retarded inhibitory effect on the movements of the intestine *in situ*

was found. This effect appeared 1 or 2 min. after the injection into the jugular vein, that is, when the pressor effect had already attained its maximum. Dilute hypertensin (1 unit in 200 c.c. Ringer solution) tested on the L  wen-Trendelenburg preparation produced a marked vasoconstriction. No change in frequency or amplitude of contraction of the toad's (*Bufo arenarum* Hensel) heart was produced even with concentrations of 1 unit in 100 c.c. Ringer solution.

*Repeated and continuous injections.* It is known that renin, vasopressin, tyramine, "veritol" and many other substances produce a lesser effect, or none at all, after repeated injections, this state being known as

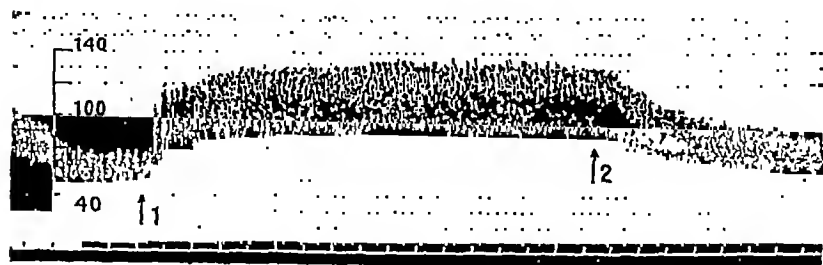


Fig. 8. Continuous intravenous injection of hypertensin. Dog, 6.5 kg., chloralosed. Double vagotomy. Artificial respiration. Femoral blood pressure in mm. Hg. Time in minutes. 1, intravenous injection of hypertensin is started at a rate of 1 unit/min.; 2, end of injection.

"tachyphylaxis". With hypertensin this does not occur. If the experimental conditions remain constant (blood-pressure level, degree of anaesthesia) the injection of hypertensin always produces exactly the same response for the same dose, even if injections are repeated at short intervals over a long time (2 hr. or more). Continuous intravenous injection of hypertensin produces an increase in blood pressure which is maintained as long as the injection is continued (Fig. 8).

The pressor effect is not modified by the destruction of the medulla, and the pithed dog has proved to be a good test owing to its low blood pressure and the constancy with which it is maintained. This shows that the action of hypertensin is peripheral and not central. Adrenalectomy does not suppress the pressor effect, although the response was smaller in dogs adrenalectomized 24 hr. before. The pressor action of hypertensin is therefore not due to a discharge of adrenaline. In an experiment after adrenojugular anastomosis hypertensin did not produce any appreciable discharge of adrenaline.

*Influence of drugs.* The previous injection of Fourneau 933 (10 mg./kg.) does not modify the pressor action of hypertensin, whereas it reverses the action of adrenaline, tyramine and urohypertensin.

The previous injection of cocaine (10 mg./kg.) and "veritol" (50-60  $\mu$ g./kg.), especially the latter, reinforces the action of hypertensin. Atropine (1 mg./kg.) has no action and pyrogallol (20 mg./kg.) slightly reinforces the action.

*The action of renin.* Renin can be easily distinguished from hypertensin by chemical methods, but their pharmacological properties are very similar. Cocaine and Fourneau 933 have the same influence on both. The increase in blood pressure, produced by a single injection of hypertensin, lasts a shorter time than that of renin but, by continuous injection, the pressure curves can be made equal.

Renin diluted with Ringer solution 1 in 50 has no vasoconstrictor action on the L  wen-Trendelenburg preparation. Horse's serum (diluted with Ringer solution 1 in 8) also has no action. But if a mixture of both is incubated for about 15 min. at 37° C. a strong vasoconstrictor effect is observed on perfusion.

The rise in blood pressure due to the injection of renin appears gradually, while that due to hypertensin has a very short latent period.

### DISCUSSION

The substance extracted from the venous blood of ischaemic kidney has the same chemical and pharmacological properties as the substance formed by the interaction of renin and blood globulins, and it can be assumed that it is the same substance, which may be called hypertensin.

We may assume that renin is an enzyme whose substrate is a blood protein belonging to the pseudo-globulin fraction, the reaction product being hypertensin.

Several facts are in favour of this interpretation. Boiled renin does not liberate hypertensin when incubated with blood globulins. If the amount of renin is increased, the yield in hypertensin does not increase but decreases. This is due to the occurrence of two simultaneous reactions: the formation of hypertensin and its disappearance, the latter being produced by renin or by some other enzyme present in the preparations we have used.<sup>1</sup>

If a constant amount of renin is incubated with variable amounts of globulins, the amount of hypertensin formed is roughly proportional to the amount of globulins.

<sup>1</sup> See note at the end of this paper.

There appears to be a certain specificity for the substrate (blood pseudo-globulins), as no hypertensin is formed if they are replaced by other proteins such as serum, albumin, haemoglobin, or by milk or egg proteins. On the other hand, renin cannot be replaced by other enzymes (pancreatin, pepsin, papain) or extracts of liver or spleen. Proteolytic activity could be revealed in our renin preparations either by the clotting of milk or by Anson's haemoglobin method [1938] as described for cathepsin. Whether renin is the same enzyme as kidney cathepsin is a problem which will require further investigation.

Some of the chemical properties of hypertensin—the insolubility in organic solvents, the inactivation by acids and proteolytic enzymes—seem to indicate that it is a polypeptide. It can easily be shown that it is different from vasopressin because it is much more stable towards acids. Its effect appears unchanged when tested on dogs treated with Fourneau 933 which leads to an inversion of the action of tyramine, adrenaline, "veritol" and urohypertensin, which can also be distinguished by chemical methods.

Does renin produce a direct increase in blood pressure, or does it act through the formation of hypertensin? In the Låwen-Trendelenburg preparation, renin has no action provided that the concentration is not too great. Blood globulins alone also have no action, but if they are previously incubated with renin, a strong vasoconstrictor action appears. Similar experiments by perfusion of a dog's tail are reported by Kohlstaedt, Helmer & Page [1938], their interpretation being that there occurs an activation of renin by blood proteins. The increase in blood pressure produced by renin is more gradual and of longer duration than that which follows a single injection of hypertensin. But it was found that by continuous injection of hypertensin a curve similar to that of renin was obtained. It is therefore more likely that both vasoconstrictor and pressor actions of renin are due to the formation of hypertensin.

Renin can be extracted from normal kidneys, but little or no hypertensin appears in the venous blood of the normal kidney; but after ischaemia hypertensin is produced and can be shown to exist in the venous blood. This difference may be due either to an activation of the enzyme or to a longer contact between enzyme and substrate.

Renin might be in an inactive state in normal kidney, the activation being produced by ischaemia or during the process of extraction. Reducing agents are known [cf. Bersin, 1935; Hellerman, 1937; etc.] to activate some proteolytic enzymes such as papain and cathepsin, and a process of reduction is quite likely to occur during ischaemia and during the extrac-

tion of renin. On the other hand, during ischaemia, the time of contact of blood with renin is probably longer and therefore hypertensin might be formed. Still another possibility is that ischaemia produces certain changes in the renal tissue, bringing renin into closer contact with the blood globulins.

### SUMMARY

The pressor and vasoconstrictor properties of the venous blood from kidneys in acute ischaemia have been studied. Extracts of this blood contain a pressor substance (hypertensin) which is also formed *in vitro* when blood proteins are incubated with renin.

Somechemical and pharmacological properties of hypertensin have been studied and found to be different from those of other known substances.

Experiments are reported which indicate that renin is an enzyme, blood pseudo-globulins the substrate and hypertensin the reaction product. Hypertensin disappears if the reaction is permitted to go too far and it is also inactivated by other proteolytic enzymes and by blood.

The pressor action of renin appears to be due to the formation of hypertensin in blood and a similar mechanism is suggested for arterial hypertension due to renal ischaemia.

The authors wish to express their thanks to Prof. B. A. Houssay for his helpful advice and criticism, and to C. Chaves and A. Bernardez for their invaluable help.

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After this paper had been submitted for publication the report of Page & Helmer, *J. exp. Med.*, 1940, 71, 29, came to our notice. Their results are, in many respects, quite similar to those we have obtained. Further experiments have shown us that ischaemic kidneys secrete renin, hypertensin being subsequently produced in the blood, and also that the destruction of hypertensin by renin is due to impurities of the preparations.

# ELECTROLYTE CONTENT AND ACTION POTENTIAL OF THE GIANT NERVE FIBRES OF *LOLIGO*

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It is usually assumed that the polarization of the bounding membrane of nerve and muscle fibres, and the consequent manifestation of a resting or "injury" potential, may be ascribed to the differential permeability of the membrane to the various species of ions which are found in the intra- and extracellular fluids. To be more precise, it is suggested that the membrane is almost impermeable to anions, very slightly, if at all, permeable to the larger cations (including Na<sup>+</sup>), but freely permeable to K<sup>+</sup> and H<sup>+</sup>. The action potential, on which the conducted impulse of the tissues probably depends [see Hodgkin, 1937, and others], is produced, then, by a momentary change in these properties of the membrane, which, during the passage of the impulse, is freely permeable to all ions. A full discussion of the theory, together with some new evidence, is given by Høber, Andersh, Høber & Nebel [1939].

Hitherto the electrical phenomena have been studied most closely in nerve, and the distribution of electrolytes in muscle, so that no really complete attempt has yet been made to discover whether the magnitudes of the resting and action potentials agree with that of the diffusion potential which might be expected from the distribution of electrolytes. Cowan [1934] estimated that the concentration of potassium was 13 times as great inside as outside the axons of the crab *Maia*. This would give a maximum possible injury potential of 64.6 mV., whereas the greatest potential which he observed was 42.1 mV. It now seems probable

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that his estimates both of the internal potassium concentration and of the injury potential are much too low. Fenn, Cobb, Hegnauer & Marsh [1934] have made careful estimates of the amount of potassium in whole nerves of frogs and Crustacea, and hence, on the assumption that all the chloride is outside the nerve fibres, of the internal potassium concentration. But the assumptions involved are so uncertain that it is not possible to accept with confidence the view of Erlanger & Gasser [1937], based on these analyses, that the internal potassium concentration is 65 times as great as the external, and able therefore to give a diffusion potential of 118 mV.<sup>1</sup>

In fact all previous attempts to establish a quantitative co-ordination between electrolyte content and action potential have met with the difficulty that, since they were made on whole nerve trunks, there was present a considerable volume of intercellular fluid, fibre sheaths and connective tissue elements interspersed among the fibres. Thus on the one hand the observed potentials were, on account of short-circuiting, less than those produced at the surface of individual fibres, and on the other hand there was introduced into the chemical analyses an error which is considerable and difficult to assess.

These difficulties may be eliminated by using the nerves of the squid, as it is possible to isolate a single giant fibre from all associated tissues (with the exception of a thin sheath), measure its potential, and then submit it to chemical analysis. This has been done for a number of fibres, and the results are presented below. It must be emphasized that the relation between action potential and potassium concentration gradient has been determined separately for each individual fibre; there has been no averaging out of results.

At the same time Hodgkin & Huxley [1939] have investigated the absolute magnitude of the action potential, especially as recorded by means of an electrode *inside* the axoplasm. The maximum potentials which they observed in this way make available for the first time for correlation with the electrolyte concentrations a reliable estimate of the value of the action potential.

Unfortunately the work was terminated by the outbreak of war, which rendered the capture of further squids impossible, so that the number of fibres dealt with is much smaller than might have been wished. Nevertheless, the results are sufficiently consistent to afford a basis for discussion.

<sup>1</sup> In any case this figure is erroneous. A concentration ratio of 65 could give rise to a diffusion potential of only 105 mV. at a temperature of 20° C.

While this work was in progress a paper by Bear & Schmitt [1939] was published, in which similar analyses of the giant fibres of a closely related species, *Loligo pealii*, were given, though without any attempt to correlate them with the action potential. On all essential points we find ourselves in agreement with their conclusions; such minor differences as exist are discussed below. The number of their analyses is far greater than that of ours, but the amount of variation in the figures for their different batches (each of which represents several fibres analysed together) would seem to indicate that experimental error is on the whole likely to be greater than errors of sampling. On the other hand it is probable that our analytical methods were the more satisfactory; the fact that Bear & Schmitt obtained average values for the K/Cl and Total base/Cl ratios in sea water of 0.0257 and 0.0296, whereas the true values are 0.0201 and 0.0312 [see Webb, 1939], suggests the possibility of systematic errors, particularly in the potassium determinations. Bear & Schmitt deduced the composition of the axoplasm by analysing first the whole fibre, and then the sheath from which the axoplasm had been extruded. Our method for correcting for the sheath is indirect. Neither method is wholly satisfactory; ours rests on assumptions which are plausible but cannot be directly proved, while that of Bear & Schmitt is open to suspicion on account of the figure (almost certainly too high) which they give for the concentration of chloride in the sheath. The volume of the sheath is, however, so small in comparison with that of the axoplasm that errors in making allowance for it are not likely to affect seriously the figures calculated for the axoplasm.

During this investigation special attention was paid to the state of the fibres before analysis, and the action potential of each fibre was tested along its whole length before it was dried for analysis. Each fibre was then analysed individually to allow of a comparison between the observed action potential and that which could be obtained as a diffusion potential from the observed potassium content.

#### METHODS

The fibres were dissected out, and the action potentials recorded at Plymouth; the chemical analyses were carried out at Cambridge. The hindmost stellar nerve was removed from a freshly killed squid (*Loligo forbesi*), care being taken to cut all branches as long as possible and to avoid kinking of the nerve. The ends were tied with fine cotton before removal, and the nerve then stretched out by means of the threads in a Petri dish filled with off-shore sea water.

The giant fibre was carefully dissected away from the smaller fibres in the nerve by means of a dissecting microscope, fine scissors, watch-maker's forceps and a very fine, sharp cornea knife. The procedure adopted was to cut transversely across a bundle of the smaller fibres close to the end of the nerve, and then, holding this bundle of small fibres with the forceps, to separate it gently from the giant fibre, cutting downwards with the cornea knife against the bottom of the dish. On reaching a point at which the giant fibre branches great care is taken to clean the branch, which must be cut *long*, otherwise the main fibre is apt to be damaged by the cut. By carefully working up and down the length of the giant fibre in this way the small fibres can be wholly removed in 2-3 hr. The best results were obtained by using a standard technique, in which the fibre was cleaned first from its upper outer edge downwards, and then from its lower inner end upwards. The final stages of the cleaning are very laborious, and small pieces of connective tissue were still left adherent even after the best dissections, especially near the points of branching. In all cases these were reduced as much as possible, and their volume must be very small in relation to that of the fibre.

After dissection the fibres were tested on the oscillograph, using an arrangement of non-polarizable electrodes similar to that described by Hodgkin [1938]. In order to avoid contamination of the surface the fibres were not raised into oil but into air. The maximum action potential of 83.5 mV. observed with this procedure is little less than the "80-95 mV." obtained by Hodgkin & Huxley [1939] by means of an internal electrode. The figures given here for the action potential are therefore likely to be only slightly less than the maximum that could be obtained from the fibres.

Except for the first two fibres studied, only those parts which gave an action potential of 60 mV. or more were used. The first two, though showing no visible signs of injury, gave only local potential responses. In the other cases lengths up to 10 cm. were obtained which gave action potentials of over 60 mV. throughout their length, and had a wet weight of up to 23 mg.

After the oscillograph record had been taken the fibres were in some cases washed for 2 min. with two changes of a sucrose solution isotonic with the sea water; in other cases they were taken direct from sea water. The significance of these two series is explained later. In either case the fibre was carefully blotted twice on filter paper, lifted at one end, and the ligature at the other end cut off with dry and very fine scissors. The severed end was allowed to fall on to a small slip of Monax glass, formed

by blowing out a thin bulb from the end of a tube and breaking it into fragments. The fibre was then laid on this slide in loops, and finally the remaining ligature cut off, the slide placed in a small Monax tube, and the whole weighed immediately on a short-arm micro-balance. The fibres were then dried for 3 hr. at  $105^{\circ}\text{C}$ . and the tube weighed once more and finally sealed off for storage and transport. The slide and tube, which had been freed from soluble material by treatment with nitric acid followed by rinsing in distilled water and steaming, were weighed together on the micro-balance before the fibre was dissected, so that by this means the wet and dry weights of the fibre were obtained. The latter was also determined again at Cambridge by weighing the glass slip with the dried fibre attached, and reweighing after the fibre had been washed off for analysis. The two determinations of the dry weight agreed, in all cases but one, to within 0.02 mg.

The following experiment shows that a short immersion in isotonic sucrose solution, though it temporarily sends up the resting potential and abolishes the irritability, brings about no irreversible change in the properties of the fibre, and may therefore be presumed not to lead to the loss of any electrolytes.

Fibre excitable in central region only.

7.15. Action potential in sea water 63 mV.

7.20. Action potential in sea water 59 mV.

Resting potential in sea water 28 mV.

7.21. Sucrose solution dropped on fibre near the electrode, sucked off and replaced by a fresh drop. Threshold rises, and fibre becomes inexcitable in 1 min. Resting potential rises steadily to 48.5 mV.

7.25. Fibre replaced in sea water. With half a minute excitability is recovered.

Action potential 56 mV.

Resting potential 28 mV.

N.B. The absolute value of the resting potential is of no significance, as it was measured with respect to the inexcitable region, which was probably not wholly "dead".

Some fibres were used for the determination of chloride; others for determination of potassium and total base.

Chloride estimations were performed by the micro-diffusion method of Conway [1935], the iodine being estimated titrimetrically against 0.05*N* sodium thiosulphate, delivered from a Rehberg micro-burette. The slip on which the fibre had been dried was soaked for half an hour in the outer compartment of the Conway unit in 1 ml. of water before the other reagents were added. Several blanks were carried out; their mean value was equivalent only to 0.4  $\mu\text{g}$ . of Cl. Trials with standard sodium chloride solution showed that the analytical error seldom exceeds 2%.

For the determination of potassium and total base the fibres were detached from the glass slips by warming them in a mixture of dilute sulphuric and nitric acids in a silica crucible of 5 ml. capacity. When most of the organic matter had been destroyed the glass slip was removed with wax-tipped forceps and washed with a fine jet of distilled water. The contents of the crucible were evaporated down, heated cautiously at 300° C. to drive off the excess sulphuric acid, and then ignited for half an hour at 800° C. in an electric furnace. At this temperature the bisulphates are completely decomposed, but there is no risk of volatilization of the sulphates. The crucible was then weighed on a micro-balance, and reweighed later after the sulphates had been washed out for the potassium determinations. From the difference in weight, which represents the weight as anhydrous sulphates of the total base present in the fibre, it is possible to calculate the total base in milli-equivalents with considerable accuracy, even without precise information as to the relative abundance of the different bases. Since the potassium is determined later the weight of potassium sulphate can be calculated. The difference between this and the total weight of sulphates may be presumed to be accounted for by sulphates of Na, Ca and Mg. One equivalent of each of these three bases yields 71.0, 68.1 and 60.2 g. of sulphate respectively. If a mean figure of 68 is taken as a basis for calculation it is extremely unlikely that the error arising from unexpectedly large quantities of Na or Mg will exceed 4% in the estimation of Na + Ca + Mg as equivalents, and therefore should not exceed 2% in the estimation of total base.

The determination of potassium was performed by the method of Robertson & Webb [1939] (precipitation as potassium silver cobaltinitrite, which is estimated by titration with ceric sulphate). The quantities and volumes of all reagents were halved, since the potassium to be estimated was only of the order of 0.15 mg. The final titration was carried out with 0.006*N* ferrous ammonium sulphate. A blank was carried out by washing out one of the Monax tubes, containing a glass slip, with a known quantity of standard potassium sulphate solution. The result was indistinguishable from a direct analysis of the same amount of potassium. Since at least two standards were analysed with each fibre the method may be considered to be free from systematic errors; fluctuating errors are usually less than 2%.

*Allowance for sheath and external film of fluid*

The sheath by which the giant fibre is surrounded consists mainly of concentric collagenous lamellae; between these and the axoplasm lie the very thin metatropic layer and a "protoplasmic" layer in which nuclei are imbedded [Bear, Schmitt & Young, 1937]. It is reasonable to assume that the bulk of this sheath becomes rapidly permeated by whatever solution bathes it; in other words that the effective barrier to permeation lies at the surface of the axoplasm. This is the basis of our method for correcting the results for the volume of the sheath and of the thin film of fluid which persists on its surface even after blotting. Two fibres (nos. 1 and 2, Table I), which had come from sucrose solution, and two (nos. 4 and 7), which had come straight from sea water, were analysed for chloride. The former gave a mean value of  $2.88 \mu\text{g.}$ , and the latter of  $4.77 \mu\text{g.}$  per mg. of total wet weight. The difference represents the chloride present in the sheath and surface film of the fibres from sea water. Since the sea water contained 19 g. Cl per litre we may calculate that for each mg. of total wet weight there is present 0.099 c.mm. of sheath plus external solution. From this the necessary corrections to the wet weight, dry weight, total base and chloride content of each of the fibres can be calculated. It is assumed that this 0.099 c.mm. of fluid is associated with 0.015 mg. of collagen, this being the approximate ratio between water content and dry weight in connective tissue.

The figures thus arrived at (axoplasm 88.5 %, sheath 11.5 % of the whole) are supported by histological data. Examination of a number of sections of giant fibres, fixed, embedded and stained in various ways, showed that the average thickness of the sheath is 6.5–7 % of the radius of the whole fibre (including sheath), and therefore that the relative volumes of the sheath and axoplasm are as 12.5 to 87.5. Fig. 2 in Bear *et al.* [1937], which is of a section cut very close to the ganglion, and selected in order to show the details of the sheath structure, is quite misleading with regard to the relative volumes of axoplasm and sheath over most of the course of the fibres. A better idea is given by Fig. 1 in Pumphrey & Young [1938].

Bear & Schmitt [1939] found that the axoplasm which they extruded represented only 82 % of the weight of the fibre. It is probable however that some axoplasm adheres to the inside of the sheath; it is also possible that they did not carry the dissection of the fibres to the maximum possible extent. There is therefore no need to assume any discrepancy between the two sets of figures in this respect.



## RESULTS

The individual analyses are set out in Table I, and summarized for comparison with those of Bear & Schmitt in Table II. It will be seen that we find a chloride concentration in the axoplasm equivalent to 20% of that in sea water, and a potassium concentration 29 times as great as that in sea water. The corresponding figures of Bear & Schmitt are 25.5% and 26 times. The only serious discrepancy lies in the figures for

TABLE I

No. of fibre	...	1	2	3	4	5	6	7	8	9
Taken from	...	Sucrose	Sucrose	Sucrose	Sea water	Sucrose	Sea water	Sea water	Sucrose	Sucrose
Wet weight (mg.)		8.078	8.222	8.814	11.105	8.650	17.677	7.425	11.722	23.4
Dry weight (mg.)		1.296	1.536	1.607	1.540	1.445	2.308	1.034	1.914	3.8
Weight of ash as sulphates (mg.)		—	—	0.226	—	0.201	0.625	—	0.323	0.6
Total K ( $\mu$ g.)		—	—	54.5	—	64.7	167.6	—	101	202
Total Cl ( $\mu$ g.)		21.4	25.5	—	45.9	—	—	40.1	—	—
Wet weight of axoplasm (mg.)		7.028	7.153	7.668	9.773	7.526	15.559	6.534	10.200	20.4
Dry weight of axoplasm (mg.)		0.971	1.207	1.254	1.318	1.099	1.955	0.886	1.445	2.9
Dry weight of axoplasm as % of wet weight		13.8	16.8	16.3	13.5	14.6	12.6	13.6	14.2	14.2
Weight of intracellular water (mg.)		6.057	5.946	6.414	8.466	6.427	13.604	5.648	8.755	17.5
Extracellular ash as sulphate (mg.)		—	—	0	—	0	0.072	—	0	—
Extracellular Cl ( $\mu$ g.)		0	0	—	20.9	—	—	14.0	—	—
Intracellular Cl (m.eq./kg. water)		100	121	—	83.2	—	—	130	—	—
Intracellular K (m.eq./kg. water)		—	—	218	—	258	314	—	295	314
Total base (m.eq./kg. water)		—	—	460	—	389	510	—	460	510
Maximum potential attainable by diffusion of K <sup>+</sup> (mV.)		—	—	78	—	82	87.5	—	85.5	87.5
Maximum action potential observed (mV.)		—	—	63	83.5	73	83.5	73	82	83.5

total base; we find this to be 20% lower in the axoplasm than in sea water, whereas Bear & Schmitt find it to be 15% higher than in sea water, though slightly lower than in the blood. It is hard to say which of these is more likely to be correct; instances may be found in the literature of analyses of the tissues of marine molluscs which reveal a total base content far higher [M'Cance & Shackleton, 1937] or far lower [M'Cance &

Shipp, 1933; Krogh, 1938a] than that of the sea water in which the animals were living. Cowan [1934] and Schmitt, Bear & Silber [1939] found the concentration of total base in the whole nerves of Crustacea to be almost identical with that in the blood and in sea water. It is probably best to assume that the same is true in *Loligo*, and that a figure intermediate between ours and that of Bear & Schmitt is correct.

TABLE II. Figures are expressed in mM. or m.eq./kg. of water

	Webb & Young		Bear & Schmitt [1939]		
	Axo-plasm	Sea water	Axo-plasm	Blood	Sea water
Cl (mM.)	109	540	130	530	510
K (mM.)	279	9.7*	310	17	12
Total base (m.eq.)	477	597*	620	670	540
Sum of ions accounted for (mM.)	585†	1102*	715†	1130†	995†
Anion deficit (total base - Cl) (m.eq.)	368	57†	490	140	30

\* Calculated from the chlorinity in accordance with the established ionic ratios.

† These figures are subject to some uncertainty, as it is not known how much of the base other than K is univalent (Na) and how much bivalent (Ca + Mg). In calculating our own figure it has been assumed that the ratio of Na to Ca + Mg is the same in the axoplasm as in sea water, so that the mean valency of the bases other than K is 1.12. The figures of Bear & Schmitt (which were given as 750, 1200 and 1050 mM. for axoplasm, blood and sea water respectively, on the assumption that all the base is univalent) have been emended accordingly. This procedure is obviously justifiable for sea water, probably also for the blood, which, judging by the data available for other molluscs [see Krogh, 1939, p. 56], is not likely to differ much in composition from sea water, and with respect to the axoplasm seems to be more plausible than assuming complete absence of bivalent bases.

‡ Of this over 55 m.eq. is accounted for by  $\text{SO}_4^{--}$ , the remainder by  $\text{HCO}_3^-$ .

It will be noticed that Bear & Schmitt provide analyses of the blood for comparison with those of the axoplasm. We have not done this, since for our immediate objective—the determination of the physico-chemical basis of the action potential—it is irrelevant; the potential is measured when the nerve is bathed not with blood but with sea water. But for general questions of electrolyte equilibria at the cell surface it is of course with the blood that the axoplasm must be compared. A difficulty lies in the fact that Bear & Schmitt do not state whether they irrigated the nerves during dissection with blood or with sea water; if, as is probable, it was with the latter, it is uncertain to what extent the composition of the fibre may have altered by reaching a new equilibrium with its changed environment. In the case of our fibres, which had always been bathed in sea water for at least 2 hr. before being dried for analysis, it might be supposed that re-equilibration was fairly complete, but it is impossible to speak with any certainty. In any case it seems that except for the calculation of the potassium ratio the differences in composition between the blood and sea water are not sufficient to give rise to any serious errors.

## DISCUSSION

(1) *Action potential.* The last two lines of Table I show that there is good agreement between the action potentials observed and the diffusion potentials which would arise if the membrane separating the axoplasm from the sea water were permeable only to potassium ions and were rendered permeable to anions during the passage of the impulse. These have been calculated from the formula  $E = \frac{RT}{F} \log_e \frac{[K^+]_{\text{inside}}}{[K^+]_{\text{outside}}}$ , which at 20° C. becomes  $E = 58 \log_{10} \frac{[K^+]_{\text{inside}}}{[K^+]_{\text{outside}}} \text{ mV.}$  The observed potentials are on the average 7.5 mV. below the calculated ones. This may be ascribed to various causes, of which the most important is probably the short-circuiting between the electrodes, which were placed, of course, on the outside of the fibres. In no case was the observed potential greater than that calculated from the potassium concentration, but it must be remembered that, using internal electrodes, Hodgkin & Huxley [1939] found potentials as high as 95 mV., that is to say greater than could be explained by any of the potassium concentrations found in the series of fibres here analysed. It would appear, therefore, that diffusion of potassium ions is the principal factor responsible for the development of the action potential, but that there may be other as yet unknown agencies which raise it by about 10 mV.

The figures quoted for the action potential denote the difference between the resting potential and the summit of the spike. After passage of the impulse the potential returns to a figure 15 mV. or more above that of the resting potential (the "positive after-potential"), and only gradually returns to its initial value. The total span of the curve may be as great as 105 mV.; to obtain this as a diffusion potential would require a ratio of internal to external potassium concentrations of 65, which is out of the question. It is clear that the process responsible for the phase of after-positivity cannot be the migration of potassium ions across the cell membrane.

(2) *The resting potential.* Hodgkin & Huxley find that the resting potential is about 50 mV. This figure is of necessity an arbitrary one, as it differs from the true potential across the cell surface by the amount of the liquid junction potential between the axoplasm and the internal sea-water electrode. The sign of this correction will clearly be such as to make the real resting potential greater than the apparent one; for the greater mobility of  $K^+$  (dominant in the axoplasm) in comparison with  $Na^+$  (dominant in the sea-water electrode) and the greater mobility of the anions of sea water in comparison with those in the axoplasm (which

are largely unknown, but almost certainly less mobile than  $\text{Cl}'$ ) will both tend to render the internal electrode positive with respect to the axoplasm. The magnitude of the correction is far more difficult to assess; ideally it should, of course, be such as to make the resting and action potentials identical, so that at the top of the curve the potential across the surface is zero. This would imply a liquid junction potential of 35–40 mV., which seems improbably high. Some more definite indication as to the nature of the missing anions in the axoplasm is necessary before any conclusion can be reached on this point.

If the resting potential arises entirely from diffusion of potassium ions it should be possible to abolish it, or even to reverse it, by raising the concentration of potassium in the surrounding solution. We have not had an opportunity of performing any experiments of this type, but Cowan [1934], working on the nerves of *Maia*, found that as the potassium content of the surrounding solution was raised the resting potential fell off, until at a concentration of 17 to 26 times the normal it vanished. This figure is approximately what would be expected from the internal potassium concentration which we and Bear & Schmitt [1939] have found in *Loligo*. Cowan's figure of 13 for the potassium concentration ratio is of course too low; since the nerve he used contains about 65% axoplasm [Young, 1936] the true ratio is presumably about 20. He found that even an external potassium concentration of 50 times the normal did not produce a reversal of potential. Cowan's explanation of this in terms of a Donnan equilibrium can hardly be adequate, since for the establishment of such an equilibrium it is necessary that the membrane should be permeable to at least one species of anion. It is more likely, as Höber *et al.* [1939] suggest, that the cytolytic effects of such high potassium concentrations render the membrane much more permeable, and thus prevent the realization of any such potential reversal.

(3) *Intracellular chlorides*. The agreement of our results with those of Bear & Schmitt leave little doubt that the axoplasm contains chlorides at a concentration of about 0.12 *M*. There is a considerable body of evidence for supposing that the cells (other than erythrocytes) of vertebrates, or at any rate of mammals, are chloride free [see Fenn, 1936, for discussion], but the indiscriminate extension of this principle has recently been contested by Krogh [1938*a*, 1938*b*] and by Conway and his school [Conway & Cruess-Callaghan, 1937; Conway & Boyle, 1939; Conway, Kane, Boyle & O'Reilly, 1939]. According to Krogh [1938*b*] "while it may be true that  $\text{Cl}$  is not normally present inside muscle fibres in mammals and perhaps even in frogs it is very doubtful if this holds for

tissues generally, even in mammals, and it is certain that it does not hold for a probably very large number of lower animals". It seems possible that the distinction lies not between higher and lower animals as such, but between those forms (vertebrates and non-marine invertebrates) in which the anionic concentration of the body fluids is sufficiently low (ca.  $0.2M$ ) to be entirely replaced inside the cell by protein, phosphates, bicarbonates and organic anions, and marine invertebrates, in which the anionic concentration of the body fluids is so high (ca.  $0.6M$ ) that inside the cell some chloride is required to make up the total. On the other hand, recent analyses of the muscle of *Loligo* [Manery, 1939] and the nerve of *Homarus* [Schmitt *et al.* 1939] suggest that in these tissues the concentration of intracellular chlorides is much less than that in the axoplasm of *Loligo*, and is perhaps little greater than that in the cells of vertebrates.

The claim of Conway and co-workers to have demonstrated the presence of chloride in the fibres of the frog's sartorius is of importance with reference to the properties of the membrane and the mechanism of potassium accumulation, but the normal concentration of intracellular chloride would appear to be so small (not more than  $4\text{ mM.}$ , and perhaps less than  $2\text{ mM./kg. water}$ ) that, in the drawing up of osmotic and electrolytic balance sheets, it may be ignored. Conway & Boyle claim that the product of potassium and chloride concentrations inside and outside the fibre are equal. It is clear from our figures that this rule does not apply in the case of our material.

(4) *Potassium concentrations in nerve and other cells.* The concentration of potassium in the axoplasm of *Loligo* seems to be as high as in any type of cell that has been investigated, though the number of reliable analyses is rather meagre. Fenn *et al.* [1934] found  $158\text{ mM./kg.}$  of whole nerve in *Libinia* (a spider crab) and  $203\text{ mM.}$  in *Homarus*, which probably implies a potassium concentration in the axoplasm of about  $300\text{ mM./kg. water}$ , approximately equal to that in *Loligo*. Cowan's [1934] analyses of *Maia* nerve, corrected on the assumption that 65% of the nerve is axoplasm [Young, 1936], yields a similar but rather lower figure of  $230\text{ mM./kg. of axoplasm water}$ . In vertebrates, where the total concentration of the serum is only  $240\text{--}350\text{ mM.}$ , the potassium content of nerve is naturally rather lower. Fenn *et al.* [1934] found  $3.0\text{--}4.8\text{ m.eq./100 g.}$  whole nerve in the frog, from which they calculate that the "potassium space" (axoplasm) contains  $176\text{ mM.}^1$  potassium/kg. water,

<sup>1</sup> Figures quoted from Fenn *et al.* [1934] and Fenn [1936], which are there expressed in terms of the total water of the tissue, have been recalculated and expressed in terms of intracellular water.

that is to say a concentration about 65 times as great as that in the plasma. Pichler's [1934] analyses of the whole central nervous system of frogs points to a similar figure for intracellular potassium. The analyses made by Alcock & Lynch [1911] of the splenic nerve of the horse do not permit of more than about 125 mM./kg. axoplasm water.

For non-nervous tissues the only figures of particular relevance are those of Manery [1939] and Bialaszewicz & Kupfer [1936]. The latter in their analyses of muscles found 190 mM. potassium/kg. in *Loligo*; this corresponds to perhaps 250 mM./kg. of fibre water. Manery's figures for the same material are, however, much lower (114 mM./kg. muscle, corresponding to about 160 mM./kg. of fibre water). Figures for vertebrate muscle are again lower (125 mM./kg. water in the "potassium space" of frog muscle, according to Fenn [1936]).

It should be mentioned that McCance & Shackleton [1937] reported very high potassium concentrations, up to 400 mM./kg. of total body water, in certain Gastropods, but did not localize the potassium in any particular tissue.

It has often been suggested that part of the potassium inside cells is "bound", that is to say non-ionized. If this were true of our material it would mean that the anion deficit would be less than appears, but on the other hand the osmotic deficit would be greater. Actually the evidence for bound potassium (discussed by Fenn [1936]) has never been very strong, and it seems best to assume that in the axoplasm it is entirely ionized. Pichler [1934] argues from the insolubility of much of the potassium of the frog's central nervous system in 96% alcohol (in which KCl is easily soluble) that less than half of it is ionized. But, as has been shown above, the chloride content of vertebrate cells is so low that only a small fraction of the potassium can be present as KCl. Furthermore, the condition in the central nervous system may be different from that in peripheral nerve, and it also seems possible that treatment with 96% alcohol may precipitate or fix the potassium in the form of compounds which do not exist in the living cell. No plausible chemical explanation of the condition of the alleged bound potassium has yet been given. The theory of Koch & Pike [1910] that most of the potassium is bound to the lipoids, particularly cephalin, can only apply to heavily myelinated fibres; in any case it has been criticized by Page [1937, p. 90], who points out that cephalin has no base-binding powers.

(5) *Anionic and osmotic deficits.* This aspect has been discussed by Bear & Schmitt [1939], and little more need be said. Our analyses imply an anion deficit of 368 m.eq. and an osmotic deficit of 735 mM.; theirs an

anion deficit of 490 m.eq. and an osmotic deficit of 280–415 mM. As is pointed out by Bear & Schmitt, bicarbonates, sulphates, phosphates, lactates, and proteins acting as acids are quite inadequate to balance the excess base, since together they could hardly amount to more than 120 m.eq. It follows, therefore, that some completely unknown anion or anions must be present at a concentration of at least 0.25 *N*. From the figures of Bear & Schmitt it would appear that at least a fraction of these anions must be multivalent; on the other hand, if ours are accepted it would be possible for them all to be univalent and still leave an osmotic deficit of 170 mM. to be filled by non-electrolytes or ampholytes. It is worth recalling in this connexion that Kelly [1904] found taurine in concentrations up to 400 mM./kg. in the muscles of lamellibranchs, and that considerable quantities of betaine are present in the muscles of *Octopus* [Henze, 1911]. Schmitt *et al.* [1939] have brought forward some evidence which suggests that in *Homarus* nerves the missing anions may for the most part be dicarboxylic amino-acids.

The only other animal cells for which balance sheets of the internal and external electrolytes have been drawn up are the fibres of the frog's sartorius and the human erythrocyte. In the former [Fenn, 1936] there is no osmotic deficit, but there is an anion deficit of about 30 m.eq. after allowance has been made for proteins. Variation in the phosphate content, however, coupled with lack of precise information as to the pH of the interior of the fibre, renders the calculations rather precarious. In the case of the human erythrocyte there is neither an osmotic nor an anion deficit, since Farmer & Maizels [1939] have recently shown that the organic phosphates are more than adequate to cover the base not bound by chloride, bicarbonate or protein.

From various fragmentary analyses scattered through the literature [see Krogh, 1939] it would seem that considerable osmotic and anion deficits, particularly the latter, are often to be found in the tissues of marine invertebrates.

#### SUMMARY

1. Action potentials have been recorded from a number of isolated and cleaned giant nerve fibres of *Loligo forbesi*, and the same fibres then subjected to chemical analysis.

2. The average concentrations of chloride, potassium and total base in the axoplasm are 109 mM., 279 mM. and 477 m.eq. respectively per kg. of water. There are large anionic and osmotic deficits. These analyses agree in most respects with those of Bear & Schmitt on the fibres of *L. pealii*.

3. The observed action potentials approximate closely to the diffusion potentials which would be produced if the bounding membrane of the axoplasm were permeable only to  $K^+$ , except during the passage of the impulse.

4. The results are compared with the available data for the electrolyte content of other types of cell.

We wish to thank Mr A. L. Hodgkin for his collaboration in the recording of the action potentials, and for helpful discussion throughout.

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THE EFFECT OF RENIN ON URINE FORMATION<sup>1</sup>BY G. W. PICKERING AND M. PRINZMETAL<sup>2</sup>*Department of Clinical Research, University College Hospital  
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IN the course of some experiments [Pickering & Prinzmetal, 1938] on renin, the protein-like pressor substance first extracted from the kidney by Tigerstedt & Bergman [1898], it was noticed that the injection of strongly pressor renal extracts often caused unanaesthetized rabbits to void abundant pale urine. In view of the implications on the function of renin which a renal action might have, it seemed worth while to investigate this point in greater detail, and this paper records the results obtained with renal extracts containing renin in preliminary experiments on anaesthetized rabbits and an unanaesthetized dog, and in more detailed observations on unanaesthetized rabbits.

The conclusion which we shall reach is that there is present in the kidney a substance which, injected into the circulation, profoundly modifies renal function. Our present evidence is that this substance is the pressor substance, renin, and we shall so term it in this paper, but it is to be mentioned that a pure preparation of this substance has not yet been obtained, and the possibility cannot be finally excluded that the renal effects are due to another constituent of kidney closely resembling renin in some physical and chemical properties.

## PRELIMINARY EXPERIMENTS

In six rabbits lightly anaesthetized with 0.15 g. sodium luminal per kg., in which a bladder cannula had been inserted an hour or more previously under ether anaesthesia, saline extracts of alcohol-dried rabbit's kidney [Pickering & Prinzmetal, 1938] in amounts corresponding to 0.5-1 g. kidney were consistently found to raise arterial pressure and accelerate the flow of urine (Fig. 1). The same extracts boiled for 5 min.

<sup>1</sup> Work done on behalf of the Medical Research Council.

<sup>2</sup> Research Fellow of the American College of Physicians.

had no effect on urine flow or arterial pressure. 4 mg. tyramine gave similar but more transient rise of blood pressure and smaller increases in urine flow.

Very similar experiments were described by Bingel & Claus [1910], who observed that in the rabbit, lightly anaesthetized with urethane, renal extracts raised arterial pressure and increased urine flow, the kidney volume rising simultaneously. The behaviour of the kidney is, however, greatly influenced by anaesthetics; we therefore proceeded to ascertain the effects of renin on an unanaesthetized dog, and in these observations we were fortunate to have the collaboration of Dr G. W. Theobald.

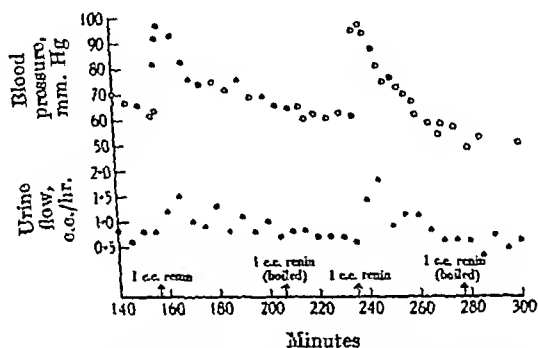


Fig. 1. Rabbit 2.0 kg. 10 Mar. 1937. 3 c.c. 10% sodium luminal injected subcutaneously and 60 c.c. water given by stomach tube at 10 a.m. Bladder cannula inserted under light ether anaesthesia at 11.30 a.m. Experiment begun at 1 p.m. Renin solution prepared by alcohol method, 1 c.c. being equivalent to 0.5 g. rabbit kidney.

The animal used was a bitch in which the urethra had been exposed by a previous perineal operation and in which urine flow was recorded from a catheter passed into and maintained in the bladder. She had been repeatedly used by Dr Theobald for the assay of posterior pituitary principle and was accustomed to the experimental procedure adopted. Adequate tissue hydration was secured by giving 250 c.c. water by stomach tube on the preceding evening and again on the morning of the experiment, before the response to renin was tested. Saline extracts of alcohol-dried rabbit's kidney, to some extent purified by half-saturation with ammonium sulphate and subsequent dialysis first against water and then against 0.9% sodium chloride, produced no increase in urine flow when injected intravenously in doses of 1-5 c.c. after the subsidence of water diuresis. Injected on the ascending limb of water diuresis 5 c.c. of this extract reduced urine flow from 250 to 15 c.c./hr., the effect lasting about 60 min. This antidiuretic action was abolished by heating the

extract for 2 hr. to 60° C., a procedure which has been previously found to inactivate renin [Pickering & Prinzmetal, 1938].

The preparation of rabbit's renin used in these experiments undoubtedly contained other protein and, to exclude the antidiuretic effect being a non-specific response to a foreign protein, extracts were made from dog's renal cortex and medulla, the former containing renin, the

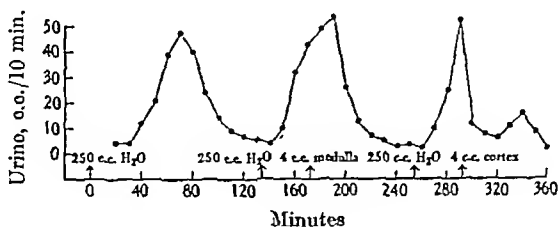


Fig. 2. Unanaesthetized bitch, 5 Jan. 1938. Urine from catheter retained in bladder. Chart showing diuresis following administration of 250 c.c. water by stomach tube, and the effects of extracts of medulla and cortex of dog kidney.

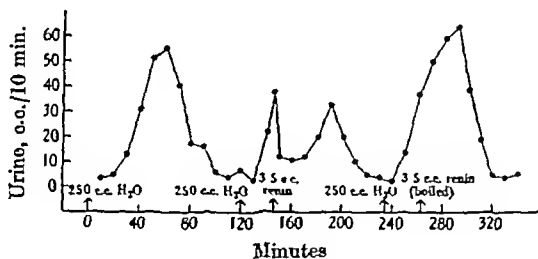


Fig. 3. Same animal as in Fig. 2. 7 Jan. 1938. Showing abolition of antidiuretic effect of cortical extract (renin) by boiling.

latter none. The fresh kidneys of a dog killed by bleeding were separated by dissection into cortex and medulla, the knife passing to the medullary side of the line of demarcation. The cortex was pulped in a mortar, treated with 2 c.c. absolute alcohol per g. for 5 hr. in the refrigerator, and filtered. The residue was dried at room temperature and the dry powder extracted with 5 c.c. 0.9% saline per g. The medulla was similarly treated. Tested on the blood pressure of an unanaesthetized rabbit, 1 c.c. of the extract of medulla was inactive, while 1 c.c. of the cortical extract produced a rise of 50 mm. Hg. The effects of these two preparations on the urine formation of the dog are shown in Figs. 2 and 3. It will be seen that 3.5 and 4 c.c. of extract made from cortex inhibited water diuresis for about 45 and 50 min. respectively, and that the effect was

abolished by boiling the solution for 5 min. No antidiuretic effect was observed from 4 c.c. extract of medulla.

Merrill, Williams & Harrison [1938] have found that renal extracts, prepared from pig's kidney by a method very similar to the alcohol method used by us, usually have a diuretic action in the unanaesthetized dog. They consider that the antidiuretic effect they occasionally observed was due to some substance other than renin in their extracts, but it is not clear on what evidence this opinion is based. In our experiments, the antidiuretic substance resembled renin in its insolubility in alcohol and in half-saturated ammonium sulphate, in its inability to pass through cellophane, in its instability at 60° or above, and its presence in cortex but not in medulla of kidney.

Although the behaviour of our dog may have been exceptional, the results are reported because they were clear-cut and because they indicate that further work in this species is desirable. It proved, however, more convenient to us to make a further investigation on the unanaesthetized rabbit.

## OBSERVATIONS ON THE UNANAESTHETIZED RABBIT

(By G. W. PICKERING)

### METHODS

Urine was obtained by catheter from adult male rabbits weighing 2-3.5 kg. and fed on a mixed diet of oats, bran and green stuff. In most rabbits a rubber catheter (size 6) proved satisfactory, but in some animals this consistently entered the seminal vesicle and in these a coudé gum elastic catheter of similar size was employed. Emptying of the bladder was ensured as a routine by massage of the hypogastrium, and by washing out the bladder with air. The bladder was washed out with 5 c.c. water in those experiments where creatinine clearance estimations were made and in a few other instances. Between catheterizations the animals were placed in wire cages small enough to make it difficult for the animal to turn round, and fitted with a drain to collect any urine which might be voided spontaneously, an event that proved infrequent.

The secretion of urine in the rabbit is greatly influenced by the degree of hydration and by the state of the alimentary canal, and we found that to obtain anything approaching consistent results it was necessary to starve the animals of food during the experiment, and for 12 hr. previously, and to allow access to about 200 c.c. of water during the night preceding the experiment; most of this was usually drunk.

*Renin.* The renin used in these experiments was a purified preparation made from rabbit's kidney in the following way. Fresh rabbit's kidney was pulped and treated with 2 c.c. of alcohol/g. for 24 hr. in the refrigerator. The residue separated by filtration was dried at room temperature, powdered and stored at 3° C. When about 50-100 g. had accumulated the powder was extracted with 10 c.c. physiological saline/g. for 24 hr. The extract was acidified to pH 4-5, and the precipitate centrifuged off. The clear yellow supernatant fluid was half-saturated with ammonium sulphate, and left for 24 hr. in the

refrigerator. The precipitate was separated by filtration, dissolved in water and dialysed through cellophane for 24-48 hr. till free from sulphate. Insoluble matter was separated by the centrifuge, and the supernatant fluid evaporated to dryness in a vacuum desiccator. A yield of about 0.7 g. of a pale yellow powder was so obtained from 250 g. fresh rabbit's kidney. This powder, kept in a sealed tube in the refrigerator, maintained its activity for several months. The dose for testing was dissolved in 1-1.5 c.c. physiological saline on the day of experiment.

The renin content of each preparation was assayed by a method previously described [Pickering & Prinzmetal, 1938] and is described in this paper in terms of units. Four preparations were used, having activities of between 0.3 and 0.6 unit per mg. One unit represents the amount of renin contained in 100 mg. of our standard power.

*Chemical estimations on urine and blood.* Chlorides were estimated in the urine by the modified Volhard-Harvey titration [Peters & Van Slyke, 1932], and in plasma by Claudius's [1922] method on 0.1 c.c. samples. Creatinine was estimated in urine and in the Folin-Wu filtrate of plasma by the colorimetric methods of Folin [1914] and Folin & Wu [1919]. Urea was estimated in the fresh urine by the urease method [Van Slyke & Cullen, 1916] and by the hypohomite method.

Blood samples of 3-5 c.c. were taken into powdered heparin from incisions in an ear vein, the plasma being separated by the centrifuge.

*Creatinine clearance.* To raise the plasma creatinine level above the accepted requisite minimum of 7 mg./100 c.c., 1-1.5 g. of creatinine in 5 or 10% solution was injected intravenously at least 30 min. before the first clearance estimation was made. Blood samples were obtained either in the middle of each period of urine collection, or at the beginning, middle and end of the total experimental period, the levels of plasma creatinine corresponding to the mid-point of urine collection being obtained from the curve relating plasma creatinine concentration to time.

## RESULTS

### *The effect of renin when the rate of urine formation is small*

In these experiments, 100 c.c. water was given by stomach tube at 10 a.m.; 4-6 hr. later, when the urine flow was falling and had reached rates between 1.0 and 15 c.c./hr., renin was injected intravenously. Urine was collected at  $\frac{1}{2}$  hr. intervals.

No definite effect on urine flow was observed from doses of 0.1-0.6 unit of renin. Doses of 1 unit or more were unfailingly and often conspicuously diuretic. Thus, 1 unit of renin increased urine flow in each of six experiments on three rabbits, the average increase being fourfold in the first and twofold in the second  $\frac{1}{2}$  hr. A total of 2.7 units (8 mg. of the preparation used), injected in two equal doses at 15 min. interval, increased urine flow to a much larger extent in each of twelve experiments on eight rabbits, the average increase being tenfold in the first, thirteenfold in the second, and twofold in the third  $\frac{1}{2}$  hr. periods after the first injection. The diuresis following 4 units (12 mg.), injected in three equal doses at 15 min., was very similar in degree and duration. The rates of urine flow observed after the injection of the larger doses of renin were often enormous, 60-100 c.c./hr. being common. While the degree of

diuresis is evidently dependent upon the dose of renin, the method as we have experienced it in the rabbit is quite unsuited for assay. For, in a single animal on different days, the size of the response produced by a given dose of a single preparation varies very considerably.

The factor in these renal extracts which is diuretic in the unanaesthetized rabbit resembles the pressor substance renin in being destroyed by boiling. Thus, the same preparation as used in the experiments just described, when boiled for 5 min., failed to give any diuretic response when injected in doses corresponding to 1 unit (three experiments on three rabbits), 2.7 units (four experiments on three rabbits) and 4 units (three experiments on three rabbits) of the unboiled solution. Boiling, however, removes protein and it seemed possible that the diuretic effects observed with the unboiled preparations were due to a non-specific foreign protein. Accordingly, an extract was made from fresh rabbit's liver by a method identical with that here used for the preparation of renin. Similar or larger quantities by weight (8 and 16 mg.) of this preparation from liver dissolved in similar volumes of saline and injected intravenously produced no diuresis in five experiments on five rabbits. Finally, in collaboration with Dr G. C. Butler, we have found that the renin preparation described can be freed of 90% of its nitrogen for a given pressor activity, by adsorption on kaolin at acid, and elution at alkaline, reaction. Such a purified preparation of renin is also powerfully diuretic in the unanaesthetized rabbit (rabbit H, Table I). This is the present evidence for the identity of the pressor and diuretic substances present in rabbit's kidney.

The urine secreted during the diuresis following renin injection is pale, and contains protein (about 0.1%). Its pH is similar to that of the urine secreted before the injection of renin. The other constituents may now be examined in detail.

*Chloride excretion.* One of the most conspicuous changes in the composition of the urine secreted after renin injection is in chloride (Fig. 4). Table I shows the changes in chloride excretion in eight experiments when a total of 2-4 units of renin were injected in two equal doses at 15 min. intervals 5-6 hr. after ingestion of 100 c.c. water. Unlike the diuresis following ingestion of water, the diuresis following renin injection is associated with a rise in the percentage of chloride in the urine, which tends to reach a value of between 0.5 and 1% NaCl. These effects on chloride excretion are abolished by boiling the solution of renin for 5 min. (Fig. 5) and are not obtained with the liver extract previously described (Fig. 6). They are still obtained when renin is further purified

by kaolin adsorption (Table I). Changes in urinary chloride thus seem to represent an integral part of the kidney's response to renin in the anaesthetized rabbit.

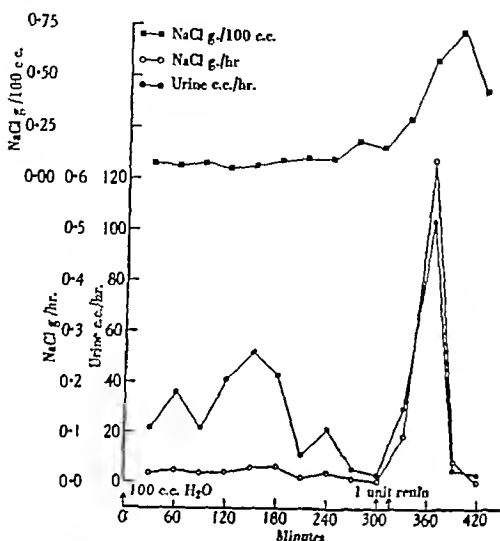


Fig. 4. Unanaesthetized rabbit H (3.2 kg.). Shows the changes in urine flow and chloride excretion induced by the ingestion of 100 c.c. tap water at 0 min. and by intravenous injection of 1 unit of renin (3 mg. of the preparation dissolved in 1 c.c. saline) at 300 and again at 315 min. In this and subsequent figures the black discs represent the rate of urine flow in c.c./hr., open circles chloride excretion in g. NaCl/hr., and black squares the urinary chloride in g. NaCl/100 c.c.

TABLE I. Showing the chloride excretion in the  $\frac{1}{2}$  hr. period before, and in the two  $\frac{1}{2}$  hr. periods after, intravenous injection of renin. 100 c.c. water was ingested 5-6 hr. previously.

Rabbit	Renin units	Urine flow, c.c./hr.		Cl. excretion, NaCl mg./hr.		Urine NaCl, g./100 c.c.	
		Before	After	Before	After	Before	After
H	2	2.6	30.4, 104	4	94, 640	0.17	0.31, 0.6
H	*	9.6	55.6, 104	4	306, 580	0.04	0.67, 0.56
193	2	15.2	20, 41.4	4	94, 210	0.03	0.47, 0.5
194	2	2.4	24, 46	8	176, 264	0.33	0.73, 0.57
191	2.7	1.4	3.8, 9.0	2	8, 72	0.14	0.24, 0.8
210	2.7	3.4	71, 78	16	414, 470	0.47	0.50, 0.6
210	4.0	3.0	29.0, 59.4	6	192, 360	0.2	0.67, 0.6
133	2.7	3.4	55, 19	24	460, 184	0.71	0.83, 0.97
133	4.0	1.8	36, 28	10	312, 260	0.55	0.87, 0.93

\* This preparation had been further purified by adsorption on kaolin, and its renin content was not precisely assayed.

The known influence of the plasma chloride level on the urinary excretion of chloride (Ambard & Weill, 1912; Aitken, 1929) suggested the

possibility that renin might act by raising the concentration of chloride in the plasma. The plasma chlorides were therefore estimated before renin and at the height of the succeeding diuresis in six other experiments made primarily to investigate creatinine clearance. The results are shown in

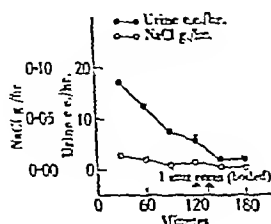


Fig. 5.

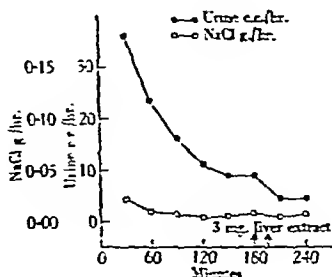


Fig. 6.

Fig. 5. The same rabbit as in Fig. 4. 100 c.c. tap water given by stomach tube 3 hr. previously. At 120 and again at 135 min. intravenous injection of 1 c.c. of a solution of renin, boiled for 5 min., the solution before boiling containing the same dose of the same preparation used in the experiment illustrated by Fig. 4.

Fig. 6. Rabbit as in Figs. 4 and 5. 100 c.c. tap water by stomach tube 3 hr. previously. Intravenous injection of 3 mg. liver extract in 1 c.c. saline at 180 and again at 195 min.

Table II. It is clear from this table that renin, in the doses here employed, produces no significant change in the plasma chloride concentration, and we may infer that the change in urinary chloride content is not a consequence of change in plasma chloride. It is also apparent that the

TABLE II. Plasma and urine chloride before and after injection of 3 units (8 mg.) renin

Rabbit	Plasma, NaCl g./100 c.c.		Urine, NaCl g./100 c.c.	
	Before	After	Before	After
R	0.63	0.68	0.11	0.68
H	0.67	0.66	0.3	0.72
133	0.67	0.65	0.48	0.68
H	0.63	0.62	0.16	0.68
211	0.58	0.58	0.09	0.62
193	0.58	0.58	0.07	0.58

chloride content of the urine after renin injection approximates to, but tends to exceed slightly, the plasma chloride. The significance of this will be discussed later.

*Excretion of sodium and other inorganic radicles.* In two experiments Dr M. Maizels and Dr A. Farmer kindly estimated the urinary content of sodium, by the method described by Salit [1932], total base, by Stadie and Ross's method [Peters & Van Slyke, 1932], and chloride. The results,



TABLE III. The content of basic and acid radicles (in milliequivalents) of urine secreted in one  $\frac{1}{2}$  hr. period before and two  $\frac{1}{2}$  hr. periods after the injection of 2.7 units renin

Rabbit	Urine specimen	Volume c.c.	Sodium		Other basic radicles		Chloride		Other acid radicles	
			m.-equiv./l.	m.-equiv.	m.-equiv./l.	m.-equiv.	m.-equiv./l.	m.-equiv.	m.-equiv./l.	m.-equiv.
133	Before renin	4.7	63	0.295	115	0.54	87	0.41	91	0.41
	After renin (1)	18	154	2.76	49	0.88	149	2.68	54	0.91
	After renin (2)	14	170	2.37	58	0.82	159	2.23	69	0.91
210	Before renin	8.3	56	0.46	9.1	0.07	23.1	0.19	42	0.31
	After renin (1)	14.5	128	1.86	13.3	0.19	114	1.65	27.3	0.41
	After renin (2)	29.7	122	3.62	5.2	0.15	103	3.06	24.2	0.71

expressed in milliequivalents (m.-equiv.), are shown in Table III, where the column "Other basic radicles" represents Total base—Na, and the column "Other acid radicles" represents Total base—Cl. From this table it will be seen that an increase in percentile and absolute excretion of Na parallels, and in both experiments slightly exceeds, the increased percentile and absolute excretion of Cl. By comparison, the changes in the excretion of other basic and acid radicles are small, and are chiefly in the direction of a fall in percentile and rise in absolute excretion, changes which may possibly be a reflexion of the increased volume of urine secreted in response to renin.

The plasma sodium was also estimated in these two experiments, and was respectively 155 and 154 m.-equiv./l. in rabbit 133, and 156 and 155 m.-equiv./l. in rabbit 210 before, and at the height of the diuresis after, renin injection. It seems clear from the experiment on rabbit 133 that the sodium content of the urine secreted in response to renin may slightly exceed that of the plasma, the behaviour of sodium, in this respect also, resembling that of chloride.

*Urea excretion.* The urea excretion in the urine was followed in seven experiments through the diuresis produced by ingesting 100 c.c. water and, after this had subsided, through the diuresis resulting from the injection of 2.5–3 units renin. The results are summarized in Table IV. It

TABLE IV. The effect of 2.5–3 units renin on urea excretion

Rabbit	Urine flow, c.c./hr.			Urea excretion, g./hr.		
	Peak water diuresis	Before renin	After renin	Peak water diuresis	Before renin	After renin
133	80	2.8	29	0.12	0.08	0.16
133	51.6	4.0	41	0.14	0.08	0.18
194	48	3.0	27.6	0.08	0.08	0.09
210	46	13.0	45.6	0.1	0.08	0.09
211	50	8.6	18.6	0.16	0.12	0.09
H	42.4	4.2	25.4	0.13	0.04	0.09
L	70	3.2	98	0.14	0.12	0.18

will be seen that the urea excretion was greater during the water diuresis than after its subsidence, and again increased during the diuresis following renin injection. There seems to be no significant difference between the increase in urea excretion produced by water and by renin, sometimes the one, sometimes the other being the greater. The conclusion drawn from these experiments is that the changes in urea excretion produced by renin are of an order to be expected from the increased flow of urine.

*Creatinine excretion.* The excretion of endogenous creatinine was followed during the waxing and waning of water diuresis and during the subsequent diuresis produced by the injection of 1-4 units of renin in nine experiments on four rabbits. Water diuresis was not accompanied by any constant or significant change in creatinine excretion. Renin diuresis, which was usually more intense than water diuresis, was accompanied by a slight but definite increase in creatinine excretion in four experiments, by no significant change in four experiments, and by a slight but definite fall in one experiment. It is evident, therefore, that the changes in creatinine excretion during renin diuresis are inconstant and often insignificant.

*Creatinine clearance.* The most obvious mechanism by which renin could act as a diuretic would be through the transmission of the rise in arterial pressure to the glomeruli, with a consequent acceleration in the rate of glomerular filtration. It is generally accepted that in most animals the glomerular filtration rate may be measured by the rate at which inulin is removed from plasma to urine. In the rabbit, though not in man, creatinine also seems to be excreted in the urine entirely through the agency of glomerular filtration, and in this animal Kaplan & Smith [1935] have shown that inulin and creatinine clearances are identical at all rates of urine flow and at all levels of plasma creatinine between 6 and 125 mg./100 c.c. We have accordingly measured the effect on creatinine clearance of renin injected during the subsidence of water diuresis.

Rydin & Verney [1938] have clearly shown the disturbing influence of strong sensory stimuli on the secretion of urine, and we experienced at first some difficulty in obtaining a regular base line of urine flow and satisfactory responses to renin in these experiments, in which the taking of blood samples introduced a considerable degree of interference. We eventually found it essential to work with animals in which the main sensory nerves to one ear had been cut, the anaesthetic ear being used for all intravenous injections and blood sampling. Urine samples were obtained by catheter at  $\frac{1}{2}$  hr. intervals, the bladder being washed out with

TABLE V. Showing the creatinine clearance in the  $\frac{1}{2}$  hr. periods before and after injecting 3 units (8 mg.) of renin, 2-6 hr. after the ingestion of 100 c.c. water

Rabbit	Urine flow, c.c./hr.		Creatinine clearance, c.c. plasma/min.		Urinary chloride, g. NaCl/100 c.c.	
	Before	After	Before	After	Before	After
H	7.4	28.0	25.3	25.7	0.16	0.68
193	20.0, 19.0	24.0	10.7, 11.3	11.3	0.09, 0.7	0.58
194	18	21.6, 18, 4.4	9.4	9.4, 8.1, 10.7	0.02	0.28, 0.11, 0.23
R	14.0	70.0	10.3	10.1	0.16	0.48
R	8.2	60.0	22.0	19.0	0.11	0.68
133	15.0, 6.0	45.8	17.2, 17.2	15.0	0.4, 0.53	0.83
210	4.4, 3.6	10.0	19.0, 16.2	18.3	0.19, 0.11	0.96

5 c.c. water. Table V summarizes the results obtained in experiments where the plasma creatinine remained above 7 mg. % during the entire experimental period; other experiments in which the creatinine had fallen below this figure gave essentially similar results but have been excluded because clearances at this plasma level would not be generally accepted as indicative of glomerular filtration rates. Kaplan & Smith [1935] have pointed out that in the rabbit the glomerular filtration rate, as measured by the inulin and creatinine clearances, varies with the rate of urine flow. It seemed, therefore, desirable to have fairly large as well as small rates of urine flow before the injection of renin, and the experiments in Table V include observations in which renin was injected at varying points on the descending limb of water diuresis from 2 to 6 hr. after the ingestion of 100 c.c. water. It will be seen that the characteristic changes in urine flow and chloride concentration were obtained in all experiments, though both changes in rabbit 194 were small, as were the changes in urine flow in rabbits 193 and 210. Nevertheless, the results in all rabbits agree in showing that when renin is injected during the subsidence of water diuresis, the increase in urine flow and urinary chloride in the succeeding  $\frac{1}{2}$  hr. is unaccompanied by any significant change in creatinine clearance.

#### *Relationship of diuretic response to blood-pressure change*

For technical reasons no determinations of blood pressure were made during those experiments in which urine was collected for measurement. As we have previously pointed out [Pickering & Prinzmetal, 1938] and since repeatedly confirmed, the response of a single unanaesthetized rabbit to a given dose of renin usually remains fairly constant from day to day, and in several animals we have therefore recorded the blood-pressure changes to the dose of renin used in the experiments on urine flow. It is to be remembered that the maximum rise in arterial pressure after

intravenous renin is reached in about 2 min., the rise then subsiding, at first rather rapidly, later more gradually, the total duration being of the order of  $\frac{1}{2}$ –1 hr., depending on the dose injected. Examples will be found in Figs. 8 and 10. 0.3 unit renin gave rises of arterial pressure of 8, 9, 11 and 18 mm. Hg in four rabbits in which this dose produced no definite diuretic response. 1 unit renin gave rises of 30, 33, 39 and 44 mm. Hg in four animals in which the same dose produced a conspicuous diuresis lasting over  $\frac{1}{2}$  and less than 1 hr., the rises of blood pressure at the end of  $\frac{1}{2}$  hr. being 7, 12, 16 and 16 mm. Hg. The conclusions we would draw from these and similar observations are that the dose of renin necessary to produce a diuretic effect is greater than that producing a detectable change in arterial pressure; and that the duration of the diuretic effect and that of the pressor effect are, within the limits of our measurement, similar. The intensity of the diuretic action does not, however, run parallel to that of the pressor effect. Thus, when 2.7 units of renin are given in two equal doses at 15 min. interval, the maximum diuretic effect is observed in the second  $\frac{1}{2}$  hr. after the first injection, whereas of course the maximum pressor effect is in the first  $\frac{1}{2}$  hr. This relationship is pursued further in the next section.

Tyramin acid phosphate injected in three equal doses, each of 2, 4 and 6 mg. at 10 min. intervals, was found to produce no consistent increase in urine flow or chloride excretion in the unanaesthetized rabbit (six experiments). These doses gave rises of blood pressure which were similar in degree to those given by renin in diuretic doses, but the rise of pressure with tyramine is of very much shorter duration (about 5 min.).

#### *The effect of renin when urine formation is high*

The results obtained in the unanaesthetized dog suggested the possibility that renin might be antidiuretic under some circumstances, and its action in the unanaesthetized rabbit was therefore investigated when given between the 45th and 60th min. after water ingestion, that is, just before the anticipated peak of water diuresis. Here again was introduced the complicating factor of disturbances in urine flow due to sensory stimuli, to obviate which we finally made our intravenous injections into an ear, the sensory nerves of which had been cut days or weeks previously. Four such rabbits gave consistent control curves of water diuresis when urine was obtained at 10–15 min. intervals, the curves being apparently uninfluenced by intravenous injections of saline, boiled renin or the liver extract described previously (Fig. 7). In all these rabbits renin produced an antidiuretic followed by a diuretic response, the antidiuretic action

being the more conspicuous with small, and the diuretic with large, doses (Figs. 8-10). Thus, 0.3 unit produced an antidiuretic effect lasting 20 min. which was followed in one rabbit only by a slight diuretic effect.

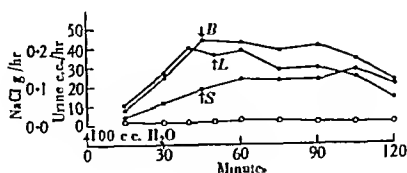


Fig. 7.

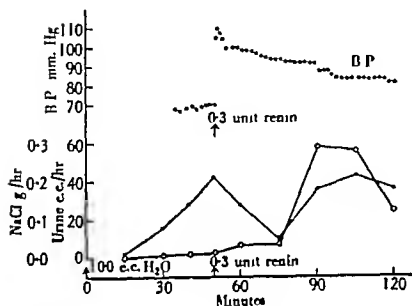


Fig. 8.

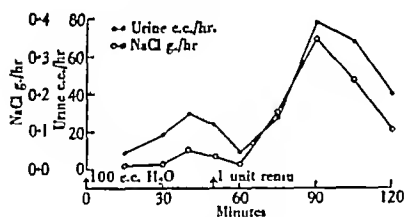


Fig. 9.

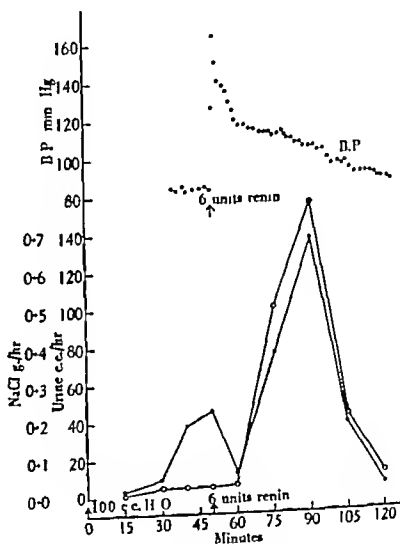


Fig. 10.

Figs. 7-10. Rabbit B (2.5 kg.). 100 c.c. of water given by stomach tube at 0 min.

Fig. 7. Three control curves obtained on separate days. Intravenous injection at B of 15 mg. renin in 1 c.c. saline, the solution having been boiled for 5 min.; at L of 15 mg. liver extract in 1 c.c. saline, and at S 1 c.c. saline. Chloride excretion corresponding to Curve L.

Figs. 8-10 Changes in urine flow and chloride excretion produced by 0.3, 1 and 6 units renin dissolved in 1 c.c. saline and injected at the 50th min. after water ingestion. In Figs 8 and 10 are shown the changes in arterial pressure produced on a separate occasion by 0.3 and 6 units renin.

One unit produced a fall in urine flow lasting 10-20 min. followed in three rabbits by a rise in urine flow above the value expected from control curves. The antidiuretic effect following 6 units was shorter, being detectable in one rabbit only when urine collections were made for 15 min.

periods, but pronounced in all when collecting periods were reduced to 10 min.; this antidiuretic effect was followed in all rabbits by a very conspicuous diuresis, urine flow frequently reaching the enormous rates of 150-180 c.c./hr.

It is evident that the threshold dose for the antidiuretic effect of renin is lower than that for the diuretic effect. Thus 0.3 unit has a definite inhibiting effect on water diuresis, but has no detectable diuretic action, be the initial rate of urine formation high or low. The diuretic action does not become conspicuous until the dose of renin is raised to 1 unit.

In every case the injection of 0.3-6.0 unit renin was followed by a rise of the chloride percentage in the urine, particularly in the specimens collected between 15 and 45 min. after the injection, the rise being observed even in those experiments in which the dose of renin was small, and the rate of urine flow never rose above the level expected from control curves. In the second 15 min. period after renin injection the plasma chlorides in three experiments were 0.69, 0.63 and 0.61 mg. NaCl/100 c.c., while the corresponding urines contained 0.64, 0.61 and 0.58 mg. NaCl/100 c.c. Again we find, therefore, that the chloride content of the urine secreted in response to renin tends to approximate to that of the plasma, but in this instance, with a large water load, urine chloride is slightly lower than plasma chloride.

In Figs. 8 and 10 are shown the changes in arterial pressure produced, in separate experiments on the same animal, by the same dose of renin used to produce the changes in urine flow. The rise of arterial pressure is greatest during the phase of reduced urine flow and continues, but is of less degree, during the succeeding phase of diuresis, or of raised urinary chloride.

It is evident from these experiments that the unanaesthetized rabbit may with suitable dosage of renin give a response similar to that which we observed in the unanaesthetized dog.

*Creatinine clearance.* Four additional experiments were made in three animals to determine the effect of large doses of renin (6 units) on the creatinine clearance, the plasma creatinine being raised to between 8 and 35 mg./100 c.c. by previous injection of creatinine. Urine collections were made during two 10 min. periods before the injection of renin at the 50th min., and for one period of 10 and one of 15 min. afterwards, blood being taken at the beginning, middle and end of the experimental period. From Table VI, which summarizes the results, it may be seen that in each experiment a profound fall in the creatinine clearance rate occurred during the phase of reduced urine flow immediately following renin injection.

TABLE VI. Urine flow and creatinine clearance, in c.c./min., before and after 6 units renin intravenously at the 50th min. following water ingestion

Min. since water ingestion	Rabbit 211		Rabbit 211		Rabbit B		Rabbit H	
	Urine flow	Creatinine clearance	Urine flow	Creatinine clearance	Urine flow	Creatinine clearance	Urine flow	Creatinine clearance
30-40	0.85	15.4	1.78	22	0.30	9.2	1.10	9.3
40-50	0.42	16.2	0.60	17	0.58	11.8	0.72	10.5
50	6 units renin intravenously							
50-60	0.28	9.6	0.10	5.5	0.15	3.9	0.20	7.0
60-75	3.00	12.0	2.60	17.8	1.10	10.0	2.70	17.8

During the subsequent phase of increased urine flow, the creatinine clearance rate was smaller in one experiment, larger in one experiment and unaltered in two experiments, as compared with the values before renin injection. These changes in creatinine clearance, corresponding to the phases of decreased and increased urine formation after renin, raise the possibility that the unchanged clearances observed in the preceding section may be the summation of an initial decreased and subsequent increased rate. Thus the results obtained in the previous section do not entirely exclude the possibility that the increased urine flow, which is the predominant effect of large doses of renin, may always be associated with some increase in the rate of glomerular filtration. This possibility does, however, seem to be excluded by the results obtained in this section on rabbits 211 and B, and particularly by the first experiment on 211 in which a urine flow at the very high rate of 3 c.c./min. was associated with a creatinine clearance considerably lower than normal. We would conclude, therefore, that the antidiuretic phase of the response to large doses of renin is accompanied by a fall in the rate of glomerular filtration, but that the diuretic phase is not necessarily associated with an increase in this rate.

*The effect of renin when chloride excretion is high*

Experiments were made on four animals, in which 100 c.c. 1.5% sodium chloride were given by stomach tube on the evening before and again on the morning of the experiment, 3-6 units of renin being injected 4-4½ hr. later. Fig. 11 exemplifies the results obtained. In all animals, the urinary chloride rose to 2.0% or more following the ingestion of saline, but there was little diuresis except in one animal. Renin produced the usual increase in urine flow, but the percentage of chloride, instead of rising, as in the other experiments, fell, rising again as the diuretic effect of renin subsided. The lowest concentrations of chloride in the urine in these experiments were found in the largest ½ hr. urine specimens, and

were in the four animals 0.75, 0.76, 0.84 and 0.86 g. NaCl/100 c.c., values which were thus very similar to those previously found for urine secreted in response to renin by animals not dosed with salt.

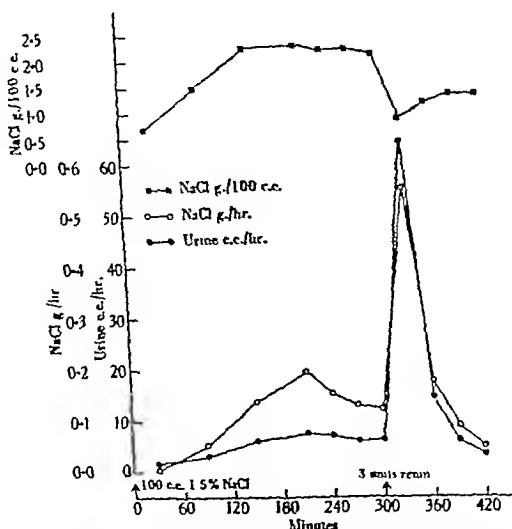


Fig. 11. Rabbit 211 (3.1 kg.) received 100 c.c. 1.5% NaCl solution on previous evening and at 0 min. 3 units renin produced increased urine flow and chloride excretion, the chloride content of the urine falling from 2.1 to 0.86% and rising again to 1.3% NaCl.

### DISCUSSION

The observations described in this paper have shown that there exists in rabbit's kidney a substance or substances which, intravenously injected into the unanaesthetized rabbit, profoundly modify the arterial pressure, the rate of formation of urine and its sodium and chloride content. Further progress in chemical separation may show that one or more of the changes in renal activity produced by our extracts are due to a substance other than that which raises arterial pressure. Particularly relevant are observations by Govaerts & Cambier [1930], who have shown that a diuresis characterized by a considerable rise in the rate of excretion of chloride is produced in the unanaesthetized dog by intravenous injection of distilled water or Witte's peptone; in both cases the animal exhibits profound somatic disturbances such as trembling, nausea, vomiting and defaecation immediately after the injection, while the diuresis does not begin until half an hour or more later. The diuresis is attributed not to any specific substance in the injected fluids but to the profound reaction in the animal which they produce. We would point



out that we have seen no signs of upset in the general well-being of our rabbits following the injection of even the larger doses of the preparations here employed; the animals sit apparently contentedly and, if permitted, move about and eat freely; they show no more than the faintest paling of the ground tone of the ears, whereas with toxic injections, intense and persistent blanching is a conspicuous feature. Nor have we been able to reproduce these changes in the volume and composition of the urine by protein containing extracts prepared similarly from rabbit's liver. If the changes in renal function which we have observed following the injection of renal extracts are not due to renin, then it seems clear that they are due to some substance which resembles it in physico-chemical properties and in its presence in kidney, and absence, or relative absence, from liver. While we must remain uncertain until chemical isolation is achieved, our present evidence favours the view that the several effects produced by our extracts on arterial pressure and renal function are due to a single substance, since they remain associated during successive stages of purification. With this qualification we may refer to these effects as those of renin.

In its powerful diuretic action, renin stands apart from other known constituents of animal tissues. Adrenaline may in small doses slightly increase the rate of urine formation in unanaesthetized rabbits [Addis, Barnett & Shevky, 1918] and in the perfused rabbit's kidney [Richards & Plant, 1922*b*], while in large doses urine flow is diminished, but there is little information concerning its effect on chloride excretion. Tyramine has been found by us to have little if any diuretic effect. Postpituitary extract resembles renin in that, in suitable doses, both may inhibit water diuresis in the unanaesthetized animal and raise the percentage of chloride in the urine [Frey & Kumpriess, 1913]. In the anaesthetized animal both may increase urine flow, and successive injections of both substances produce diminishing rises of arterial pressure. Fromherz [1923] and McFarlane [1926] claim that under certain circumstances pituitrin may increase urine flow in the unanaesthetized animal, but the effects they obtained were small, and Motzfeldt [1917] observed only an inhibition of urine flow in the unanaesthetized rabbit with intravenous injections varying between 2<sup>nd</sup> and 2 c.c. of a post-pituitary extract. From their effects on urine formation, it may be concluded therefore that renin and the post-pituitary principle are not identical, a conclusion which is supported by differences in chemical properties and in their effects on the ear vessels of the unanaesthetized rabbit [Landis, Montgomery & Sparkman, 1938].

*The nature of renin's action on the kidney.* During the few minutes immediately following the injection of renin, a phase in which urine flow is diminished, a great rise in arterial pressure is accompanied by a fall in the rate of glomerular filtration, at least when the dose of renin is large. A fall in the rate of glomerular filtration, despite raised arterial pressure, indicates constriction of the afferent glomerular arteries or diminution of the number of open glomerular capillaries, effects which we may infer are due to the action of renin on these vessels. It is to this fall in the rate of glomerular filtration that we would attribute the diminished rate of urine formation which immediately follows the injection of renin.

The increased secretion of urine which follows the inhibitory phase and which is the predominant effect of large doses of renin in the unanaesthetized rabbit cannot, on the evidence submitted here, be attributed to increase in the amount of the glomerular filtrate, in so far as this may be inferred from the creatinine clearance rate. It must therefore be attributed to a diminished reabsorption of water from the renal tubules. This is not the only change in tubular activity, since the composition of the urine during renin diuresis contrasts sharply with that during water diuresis in its sodium and chloride contents. Evidently there is also, under ordinary circumstances, a diminished tubular reabsorption of sodium and chloride. Diminished absorption of sodium chloride in the tubules would be expected by its osmotic action to diminish water reabsorption also, and we considered at one time the possibility that the increased water excretion was simply a secondary effect of diminished reabsorption of salt. This possibility has, however, been excluded by the experiments in which 1.5% saline was ingested before the injection of renin and in which the chloride concentration of the urine fell during renin diuresis. Evidently reabsorption both of water and of sodium chloride is inhibited.

Renin in diuretic doses has a pronounced and prolonged pressor effect in the unanaesthetized rabbit, and it is interesting to note that the composition of the urine during renin diuresis has many of the properties of the urine in the pressure diuresis observed in the isolated kidney. Thus, the urine during renin diuresis tends to approximate in composition to the plasma from which it is formed, at any rate so far as chloride, urea and creatinine are concerned, and these are the only constituents fully studied. The proposition that renin diuresis represents a pressure diuresis may now be considered in greater detail.

The absence of any increase in the rate of glomerular filtration, despite the presence of raised arterial pressure during the diuretic phase of renin's

action, indicates that the renal vessels are not unaffected by renin at this stage, but undergo constriction. And it may be suggested that during this phase there is a total closure of some of the originally open glomerular tufts; while in those remaining open intra-glomerular arterial pressure is greatly increased, partly by increased systemic pressure and partly by constriction of the efferent glomerular arterioles. This conception receives support from recent experiments on the unanaesthetized dog by Corcoran & Page [1939], who conclude that renin decreases renal blood flow and increases intra-glomerular arterial pressure either by increasing general arterial pressure or by efferent arteriolar constriction. Evidence for efferent arteriolar constriction has previously been obtained in dogs anaesthetized with nembutal by Merrill *et al.* [1938], who observed decreased renal blood flow and swelling of the kidney in response to renin. Friedman, Abramson & Marx [1938] in similar preparations observed a preliminary fall followed by a rise in kidney volume following renin injection.

While it might be possible on this basis to consider renin diuresis as a form of pressure diuresis in which relatively few nephrons participate, a real difficulty is encountered when we consider more closely the chloride content of the urine. Thus in the isolated kidney, when the concentration of chloride in the urine is initially low, a rise of arterial pressure is accompanied by a rise in the concentration of chloride in the urine towards, but not up to, its concentration in the blood [Richards & Plant, 1922*a*; Starling & Verney, 1925]. In renin diuresis there is a similar rise in chloride concentration, but this reaches and may surpass the plasma chloride level. It is difficult to know how much importance to attach to this difference, since the comparison is between the behaviour of the intact animal and that of the isolated kidney, and for this reason no final decision may be taken. Nevertheless, the disparity between the behaviour of urinary chloride in the renin diuresis on the one hand, and in the pressure diuresis in the isolated kidney on the other, suggests that renin may not act purely on the renal vessels, but may directly influence the renal tubule cells, inhibiting the reabsorption of water, of sodium and of chloride.

Starling & Verney found identical chloride contents in plasma and the urine secreted by an isolated dog's kidney after tubular action had been abolished by cyanide. Westfall, Findley & Richards [1934] have found identical chloride contents in plasma and glomerular urine of the frog and necturus when backflow from the tubules was prevented, though in earlier experiments when this precaution was not taken, a tendency

was observed for glomerular urine to have a higher chloride content than plasma. More recently Walker, Hudson, Findley & Richards [1937] have found, both in the frog and necturus, that the chloride content of the urine in the proximal convoluted tubule is about 10% higher than in the plasma, while that in the distal tubule becomes progressively lower. In view of the technical difficulties of the experiment these workers did not feel justified in trying to explain this difference between plasma and proximal tubule urine, but it would seem possible from their findings that the urine secreted after renin represents, as far as its chloride content is concerned, an approximation to that of the proximal convoluted tubule rather than to that of the glomerular capsule.

*General.* The existence of a substance apparently present in normal renal cortex and capable of profoundly influencing renal function may help to explain some otherwise obscure chapters in the physiology of the kidney. Recent work has shown that, in some respects at least, the behaviour of the kidney is independent of its nervous connexions and is determined by the concentration in the plasma of substances which it excretes, and by chemical substances elaborated elsewhere in the body, for example in the posterior lobe of the pituitary and in the suprarenal cortex. Exactly how these various influences are integrated, and what part, if any, renin plays in the final product are questions as yet unanswered. In this connexion it is relevant to consider dosage. It has been found in other experiments in progress in this laboratory that the renin content of rabbit's kidney varies between about 0.4 and 5.0 unit/g., a very common value being 1 unit/g. If renin exerts its diuretic action solely by its effects on the general arterial pressure and glomerular vessels, then it would presumably act by being released into the general circulation, and the experiments here described indicate that the amount released would have to be of the order of 1 unit. An average pair of rabbit's kidneys weighing 15 g. would thus contain only about 15 times the diuretic dose. If, however, renin acts directly on the tubule cells, then its presence in normal renal cortex might enable it to act on the kidney without entering the general circulation, and the amount requisite to change renal activity would be small; in this case also it might be possible for renin to affect the activity of the kidney without influencing the arterial pressure.

#### SUMMARY

1. In the anaesthetized rabbit, saline extracts of alcohol dried rabbit's kidney raise blood pressure and accelerate urine flow. Both effects are abolished by boiling the extracts.

2. In the hydrated unanaesthetized rabbit with a small initial rate of urine flow, renal extracts containing renin, in doses of 1 unit or more, produce a conspicuous diuresis lasting about an hour. The diuresis is not accompanied by any significant change in the excretion of endogenous creatinine or in the rate at which injected creatinine is cleared from the plasma; changes in urea excretion are similar to those occurring in water diuresis. There is, however, a very large increase in the excretion of sodium and chloride, the percentage of chloride in the urine tending to rise to, and slightly to exceed, that of the plasma.

3. In the unanaesthetized rabbit, renin injected on the ascending limb of the curve of water diuresis produced a transient inhibition followed by an increase in the flow of urine. With doses of less than 1 unit, the anti-diuretic effect alone may be observed, but with larger doses the diuretic effect is the more conspicuous. The rate at which injected creatinine is cleared from the plasma is decreased during the phase of diminished urine flow, and is not constantly or significantly altered during the phase of increased urine flow. The chloride content of the urine during the diuretic phase rises towards that of the plasma.

4. When in the unanaesthetized rabbit the chloride content of the urine is artificially raised by 1.5% saline ingestion, renin produces an increased urine flow accompanied by a fall in the percentage of chloride in the urine towards that of the plasma.

5. Injected intravenously, the dose of renin necessary to produce diuresis is greater than that producing a rise of arterial pressure.

6. The antidiuretic action of renin in the unanaesthetized rabbit is attributed to its action on the glomerular vessels reducing the rate of glomerular filtration.

7. The diuretic action of renin in the unanaesthetized rabbit is due to an inhibition of tubular reabsorption of water, sodium and chloride. This may represent the effects of a pressure diuresis in which relatively few nephrons are involved, but it is more probably due in part to a direct action of renin on the activity of the renal tubule cells.

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## THE MECHANISMS OF DILUTION DIURESIS IN THE ISOLATED KIDNEY AND THE ANAESTHETIZED DOG

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THE important contributions to the theory of renal secretion made by students of dilution diuresis began with Starling's [1899] consideration of the influence of the colloid osmotic pressure of plasma proteins on the rate of glomerular filtration. Despite Magnus's [1900] impression, based on extensive observations, that the diuresis was too great to be attributed solely to a change in glomerular activity, the view that tubular activity was not directly affected gained ground and was supported by Barcroft & Straub's [1910] finding that dilution diuresis was unaccompanied by increase in metabolism of the kidney and was therefore a "mechanical" diuresis. This view was reinforced by Knowlton's [1911] comparison of the effects of saline with gum-saline and gelatine-saline solutions, which identified the diuretic stimulus as a reduction in colloid osmotic pressure of the plasma. The purely "mechanical" view of dilution diuresis was strongly advocated by Cushny [1917] and has since been generally accepted as evidence in favour of the filtration theory of glomerular function in the mammal.

Cushny [1917] concedes that "the change in the amount of urine in Magnus's experiments is out of all proportion to the change in the concentration of the colloids", but he felt able to explain away the discrepancy on the grounds that the proportion of filtrate reabsorbed in the tubules would be lower during the diuresis, and that this would magnify the change in glomerular filtration rate which he apparently took to be proportional to the reduction in concentration of plasma colloids.

More recently, observations on perfused kidneys have directed attention to the disproportionately great diuresis produced by a small reduction in colloid osmotic pressure of the plasma in comparison with that

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produced by an equivalent rise in arterial pressure [Winton, 1937], with the implied suggestion that dilution diuresis is in part due to a direct action on tubular activity, inhibiting reabsorption of water. It is the main purpose of the experiments described below to measure this disproportion in the anaesthetized dog and in the isolated kidney, and to attempt to evaluate the glomerular and tubular factors co-operating in the production of dilution diuresis.

The decision as to whether a form of diuresis is to be attributed to change in glomerular or tubular activity encounters a difficulty which is simply explained by an illustration. Suppose we may measure the glomerular filtration rate in terms of the creatinine clearance, and that a twofold diuresis involves an increase of urine flow from 1 to 2% of the filtration rate. If the amount of water reabsorbed in the tubules remains unchanged, we should expect only 1% increase in creatinine clearance, a change too small to detect with confidence in view of the other hazards of biological experiment. It would seem difficult to discover, therefore, whether even a considerable diuresis is due to a small increase in glomerular filtration rate or to a small diminution of reabsorption in the tubules. Fortunately, the difficulty can be surmounted if the meanings attached to "change in glomerular activity" and "change in tubular activity" or more shortly "glomerular diuresis" and "tubular diuresis" are properly chosen and precisely defined. As far as we know, a change in glomerular filtration rate always involves characteristic and determinate changes in tubular activity, and glomerular diuresis should, therefore, be taken to imply the whole complex of glomerular and tubular changes consequent upon an increase in filtration rate. Similarly, tubular diuresis implies the changes occurring when diuresis is unaccompanied by increase in filtration rate.

To assign a particular type of diuresis to the glomerular or tubular class requires standards of reference. The standard here chosen for a glomerular diuresis is the change in a number of the constituents of urine produced by an increase in renal arterial pressure, and the standard chosen for a tubular diuresis is the corresponding change produced by increase in the urea content of the plasma. It is a further aim of this communication to give rather more formal precision to the ideas underlying this way of distinguishing between changes in glomerular and tubular activity, though the ideas themselves have been implicit in several earlier communications [e.g. Verney & Winton, 1930].

The mechanism of dilution diuresis is particularly concerned with the difference between the glomerular capillary pressure and the intracap-



sular pressure, and in suitable circumstances [Winton, 1931*a*] changes in the latter can be estimated if the pressure fall from the glomerular capsule to the ureter be kept approximately constant by preventing changes in urine flow by appropriate changes in ureter pressure. We have, therefore, compared dilution diuresis with the standard forms of diuresis not only when the increase in urine flow is allowed to become manifest, without intentional changes in pressure head, i.e. "isobaric diuresis", but also when the urine flow is kept constant by suitable adjustment to the pressure head, and the "isorrheic diuresis" is measured in terms of the change in arterial or ureter pressure.

### METHODS

The technique employed in experiments with the double pump-lung-kidney preparation was the same as that described in our previous communication [Eggleton, Pappenheimer & Winton, 1940*a*]. The chemical methods were also as there described.

Observations on the kidney *in situ* were conducted on dogs anaesthetized with chloralose, 0.1 g./kg. Ureters were cannulated, and in some experiments the left kidney was denervated. The pressure in the renal artery was varied by adjusting a clamp placed on the thoracic aorta. This and certain details of procedure are described in more detail by Shannon & Winton [1940].

Dilution of the blood was achieved by adding a Ringer-Locke's solution containing NaCl 0.76%, KCl 0.042%,  $\text{CaCl}_2$  0.024%,  $\text{MgCl}_2$  0.0005%,  $\text{NaHCO}_3$  0.20%, dextrose 0.05%. The higher value of bicarbonate than is usual was chosen in pump-lung-kidney experiments so that an appropriate pH would result from ventilation of the lungs with 5%  $\text{CO}_2$ . The degree of dilution of the plasma was estimated by measuring the total solids.

Two or three consecutive samples of urine were collected under any given set of conditions after an appropriate time had been allowed for the composition to reach a steady value. It was observed that the usual procedure of allowing a definite volume of urine, say 3 c.c., to be formed in the hope that such a "wash-out sample" would be sufficient was unsatisfactory at high urine flows—the volume of the "wash-out sample" being greater the higher the urine flow. At high rates of flow we often discarded 10 c.c. or more before beginning the collection of test samples.

## RESULTS

*The comparison of dilution diuresis with glomerular and tubular diuresis under isobaric conditions*

*Isolated kidney.* The disproportion between the degree of diuresis due to blood dilution and that due to an arterial pressure change which might be expected to produce about the same change in glomerular filtration pressure is shown in Table I. As a first approximation, the glomerular

TABLE I. Diuretic action of the changes in arterial pressure and in plasma protein concentration which may produce comparable changes in glomerular filtration pressure

	Percentage increase in urine flow due to	
	Pressure diuresis (per 5 mm. Hg change in arterial pressure)	Ringer diuresis (per 3 mm. Hg change in colloid osmotic pressure)
Isolated kidney	21.1 $\pm$ 3.5 (9)	136 $\pm$ 20 (7)
Anaesthetized dog (denervated)	12.0 $\pm$ 1.5 (2)	145 $\pm$ 31 (10)
Anaesthetized dog (innervated)	12.3 $\pm$ 4.0 (8)	212 $\pm$ 30 (20)

capillary pressure in the isolated kidney may be taken as 60% of the arterial pressure [Winton, 1931b], a reduction of 1% (e.g. from 8 to 7%) in the serum protein concentration would imply a reduction of colloid osmotic pressure of about 3 mm. Hg, and the equivalent rise in arterial pressure should, therefore, be about 5 mm. Hg. If this were so, the disproportion between pressure and dilution diuresis would be six- to sevenfold.

More precisely, the relevant osmotic pressure is the properly calculated average of that in the serum entering the glomerular capillary, and that in the serum as it leaves the capillary deprived of the glomerular fluid. If this be taken into account, the reduction of colloid osmotic pressure effective in glomerular filtration corresponding with a change by 1% in the serum protein concentration may fairly be put at 4 mm. Hg and the equivalent increase in arterial pressure should, therefore, be about 6 mm. Hg. This consideration, however, only reduces the disproportion between dilution and pressure diuresis to one of fivefold.

A first approach to the problem whether the disproportionate magnitude of dilution diuresis is due to an unexpectedly large increase in glomerular filtration or to a direct influence on the tubules reducing their reabsorptive activity may be made by comparing the data represented in Figs. 1-3. The curves illustrate the changes in urine flow, and in the

chloride, creatinine and urea clearances characteristically associated with diuresis due to (1) dilution of serum, (2) increase in arterial pressure, and (3) increase in the urea content of the serum. The figures represent observations on different kidneys. The experiment on pressure diuresis (Fig. 2)

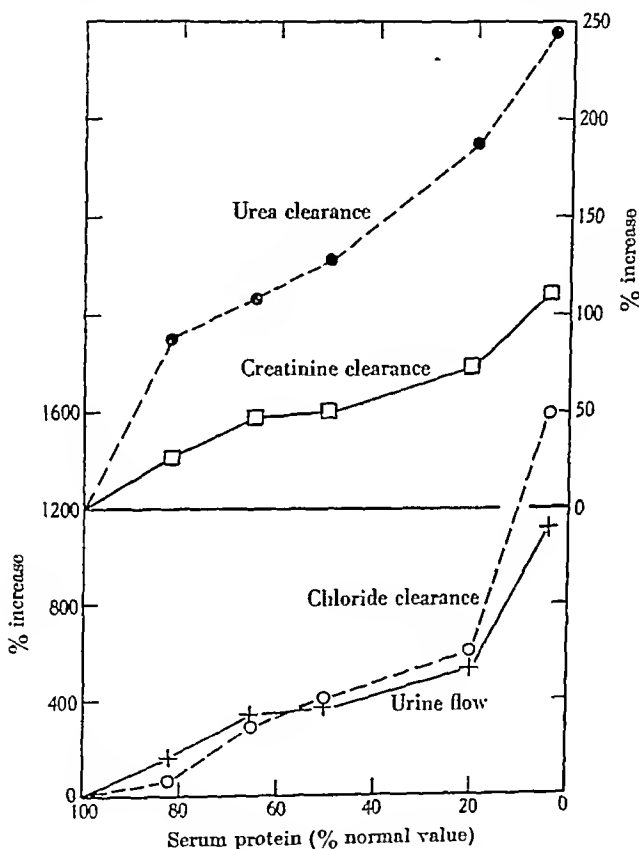


Fig. 1. Dilution diuresis in the isolated kidney. The percentage increase in urine flow, and in creatinine, urea, and chloride clearances with increasing dilution of serum protein by Ringer's fluid. Double pump-lung single kidney preparation. Initial values: kidney weight 15.5 g., urine flow 0.18 c.c./min., creatinine clearance 8.7 c.c./min., urea clearance 2.44 c.c./min., chloride clearance 0.029 c.c./min.

was performed on the single pump-lung-kidney preparation. In the other experiments (Figs. 1, 3), each kidney was perfused alternately from two pump-lung circuits, the control circuit containing defibrinated blood the composition of which was kept as far as possible constant throughout the experiment, the second circuit being used for the addition of the diuretic

agent in several stages. The blood in a double pump-lung single kidney preparation was well mixed, the two circuits only being separated shortly before the transfer of the kidney from the control circuit to that in which either Ringer's solution or extra urea had been added to the defibrinated blood.

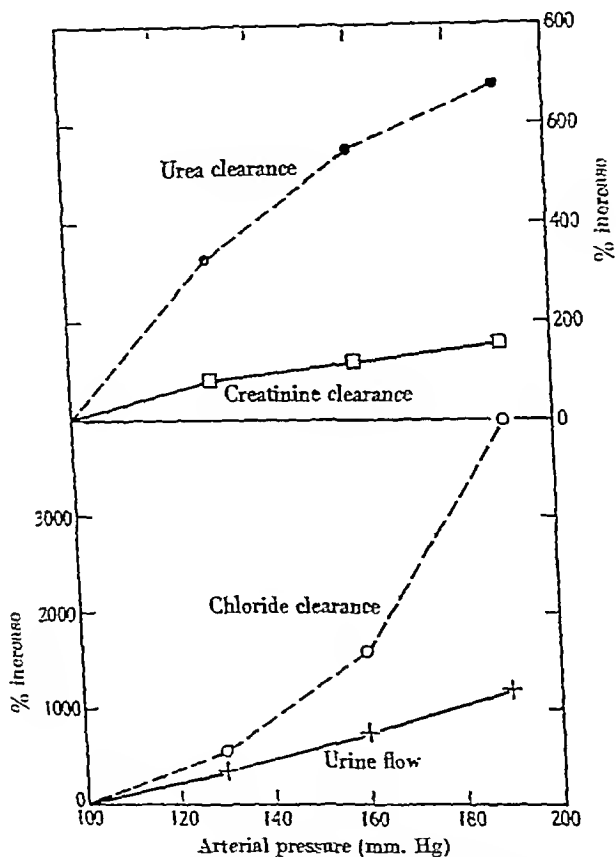


Fig. 2. Pressure diuresis in the isolated kidney. The percentage increase in urine flow, and in creatinine, urea, and chloride clearances with increase in arterial pressure. Single pump-lung-kidney preparation. Initial values: urine flow 0.092 c.c./min., creatinine clearance 5.4 c.c./min., urea clearance 0.59 c.c./min., chloride clearance 0.0019 c.c./min.

Any point on the curves represents the increase in value when the kidney was on the diuretic circuit over the average of the two values obtained just before and after, when the kidney was on the control circuit. Inspection of Figs. 1-3 immediately shows that dilution diuresis is much more closely related to pressure diuresis than it is to urea diuresis.

In both dilution and pressure diuresis the creatinine clearance increases, and the urea clearance increases relatively more. The increase in chloride clearance is less marked in dilution diuresis than in pressure diuresis, but in both there is a systematic increase which contrasts with the more complicated relation shown in urea diuresis.

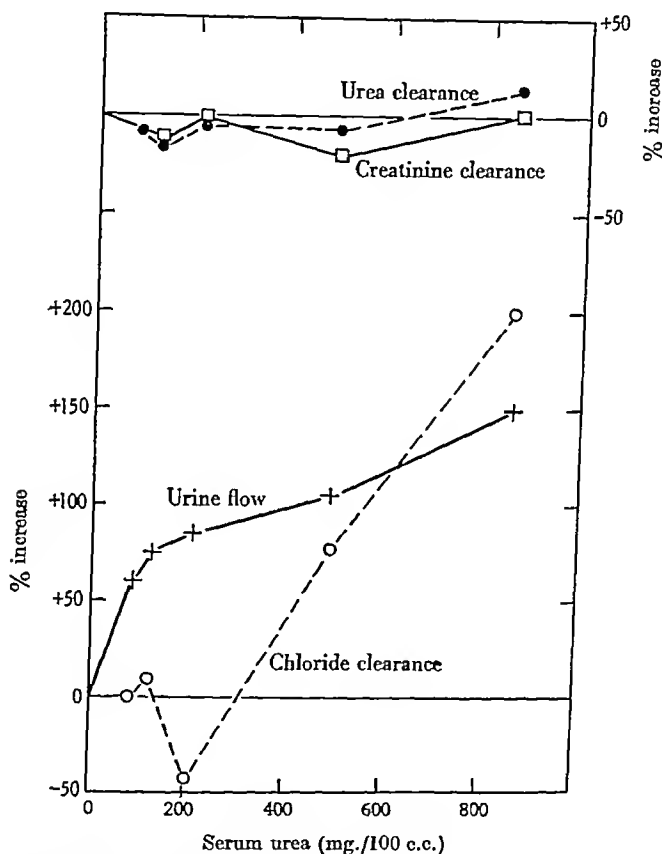


Fig. 3. Urea diuresis in the isolated kidney. The percentage changes in urine flow, and in creatinine, urea, and chloride clearances with increasing serum urea concentration. Double pump-lung single kidney preparation. Initial values: kidney weight 23 g., urine flow 0.307 c.c./min., creatinine clearance 12.5 c.c./min., urea clearance 4.2 c.c./min., chloride clearance 0.0006 c.c./min.

In Table II we have brought together for quantitative comparison a series of values from a considerable number of experiments of the same kind. We have calculated all values for a doubling of urine flow assuming proportionality between the increase in the value and the increase in the

TABLE II. Isolated kidney. Isobaric diuresis. Showing the percentage changes in clearances, etc., calculated for a doubling of urine flow by different diuretic agents

Percentage increase in	Arterial pressure	Ringer's fluid	Urea
Creatinine clearance	$31 \pm 8$	$24 \pm 7$	- $5 \pm 4$
Urea clearance	$60 \pm 10$	$45 \pm 5.7$	- $1 \pm 4$
Chloride clearance	$220 \pm 50$	$90 \pm 13$	$63 \pm 16$
Total osmotic work	$38 \pm 3$	$41 \pm 4$	$63 \pm 7$
Number of observations	9	7	8
Mean percentage increase in urine flow	$183 \pm 24$	$396 \pm 130$	$132 \pm 51$

urine flow. It will be seen from the curves given that such an assumption is only an approximation. Each value is expressed in terms of the mean, and the standard error of the mean. The increases in urine flow actually observed are included in the table, and the fact that the Ringer diuresis was considerably greater than the two other forms of diuresis should not be entirely ignored in a comparison of the other values shown.

The salient features of the results expressed in Table II are (1) the increase in creatinine clearance in dilution diuresis and in pressure diuresis is considerable and not very different in amount, whereas, by contrast, there is no such increase in urea diuresis. Considerable urea diuresis usually involves a small reduction in creatinine clearance due to increased intrarenal pressure [Winton, 1933], and comparable with the reduction of 18% found [Bickford & Winton, 1937] when water reabsorption is blocked by cooling the kidney. (2) In view of the greater increase in urine flow in the dilution diuresis, the increase in urea clearance is probably rather greater in pressure diuresis than in dilution diuresis, there being no increase in urea diuresis. The lessening in reabsorption of urea with diuresis is, therefore, on the average less marked with dilution than with pressure diuresis. (3) Increases in chloride clearance occur in all three forms of diuresis, the increase being larger in pressure diuresis. Dilution diuresis differs from pressure diuresis in promoting the reabsorption of chloride.

The evidence of Table II as to whether dilution diuresis is to be regarded as "glomerular" or "tubular" is, therefore, conflicting. It appears to be mainly glomerular with reference to the creatinine and urea clearances, but mainly tubular with reference to chloride clearance. As far as the elimination of water is concerned, however, and the problem of the disproportionately great dilution diuresis is primarily concerned with this constituent of the urine, the creatinine clearance is the surest guide to the mechanism involved, and this would suggest that in the isolated kidney dilution diuresis is mainly a glomerular diuresis. This suggestion

is supported by our previous finding [Eggleton *et al.* 1940a] that the increase in total osmotic work done in pressure and dilution diuresis is not significantly different, whereas that in urea diuresis is usually somewhat higher. This involves the conclusion that a given change in colloid osmotic pressure of the serum produces a much greater change in glomerular filtration rate than does a comparable change in hydrostatic pressure across the glomerular membrane, i.e. that either the permeability or the surface area of the glomerular membrane has undergone substantial increase.

*Anaesthetized dog.* The disproportion between dilution diuresis and the pressure diuresis due to a change in hydrostatic pressure comparable with the change in colloid osmotic pressure is even greater in the anaesthetized dog than in the pump-lung-kidney preparation. If a change in arterial pressure of 5 mm. Hg be regarded as equivalent to a change by 1% in the concentration of serum protein, as above, dilution produces about fifteen-fold the diuresis produced by an equivalent change in hydrostatic pressure (Table I). We have separated our results on innervated and denervated kidneys in Table I to indicate that they are sufficiently alike to justify considering them together in Table III. If, for reasons given in the previous section, the arterial pressure equivalent of a change by 1% in serum protein be taken as 6 mm. Hg, the disproportion in diuresis is reduced to one of about thirteenfold, which still implies a major discrepancy in the theory of dilution diuresis currently adopted.

The plan of the experiments on anaesthetized dogs from which the data in Table III were obtained was less elaborate than that adopted in

TABLE III. Anaesthetized dog. Isobaric diuresis. Showing the percentage changes in clearances, calculated for a doubling of urine flow by different diuretic agents

Percentage increase in	Arterial pressure	Ringer's fluid	Urea
Creatinine clearance	14 $\pm$ 3	4 $\pm$ 3	- 10 $\pm$ 2
Urea clearance	35 $\pm$ 2	20 $\pm$ 5	1 $\pm$ 1
Chloride clearance	475 $\pm$ 70	400 $\pm$ 70	52 $\pm$ 30
Number of observations	10	30	4
Mean percentage increase in urine flow	107 $\pm$ 44	439 $\pm$ 87	249 $\pm$ 30

connexion with Table II; it is illustrated in Fig. 4 and involves following the progressive changes due to successive doses of the diuretic agent without the intermediate returns to control conditions rendered possible by the double perfusion arrangement. Such a procedure has greater justification in the anaesthetized dog, for under our experimental conditions the urinary output and composition remain fairly constant if not

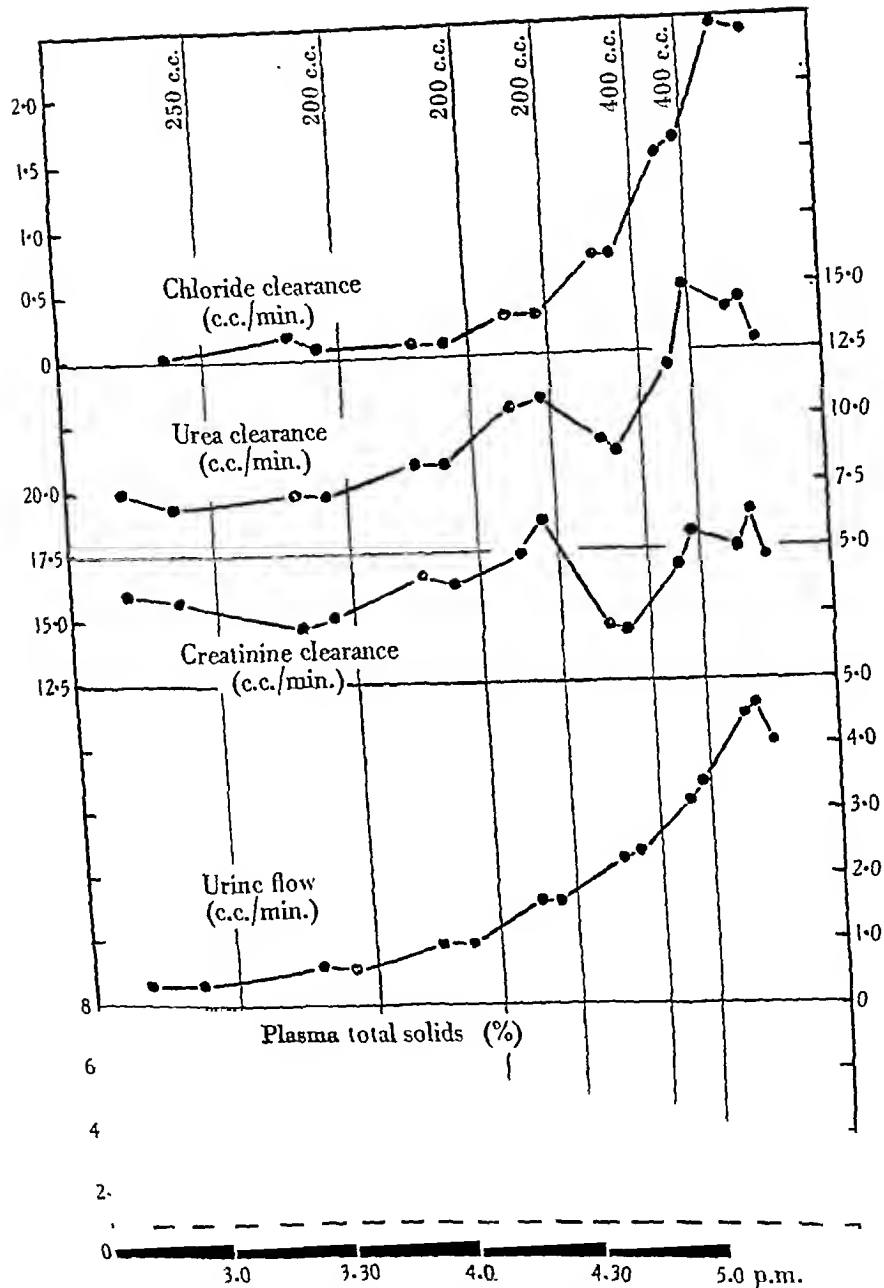


Fig. 4. Dilution diuresis in the chloralosed dog. The increase in urine flow, and changes in the creatinine, urea, and chloride clearances with decreasing concentration of serum protein due to addition of Ringer's fluid. A broken line is inserted at 1% total solids, so that the values above this indicate the approximate concentration of plasma protein. Dog 10.5 kg. Kidney denervated. Arterial pressure increased from 95 mm. Hg to 110 mm. before 3.30 p.m. and was kept approximately at that value thereafter by appropriate removal of blood.



intentionally changed as indicated in Fig. 7, whereas the output of the isolated kidney systematically declines as the experiment progresses as indicated in Fig. 6. The characteristic effects of dilution diuresis are seen in Fig. 4 and in more quantitative form in Table III. In contrast with the isolated kidney, the kidney *in situ* responds to dilution of the plasma with a change in creatinine clearance which is definitely smaller than that associated with the same increase in urine flow produced by raising the arterial pressure. In our few observations on the action of urea in the anaesthetized dog secreting under isobaric conditions the creatinine clearance decreased, and it might be fair in respect of this variable to place dilution diuresis about half-way between pressure diuresis and urea diuresis, although dilution diuresis produced no significant increase in creatinine clearance. The fall in creatinine clearance during urea diuresis may well have been due to a rise in intrarenal pressure, and a similar rise may have prevented a greater increase in clearance during dilution diuresis. It may be recalled that pressure diuresis differs from urea diuresis in being unaccompanied by a change in intrarenal pressure [Winton, 1936].

The urea clearance increases in dilution diuresis by something of the order of one-half the increase in pressure diuresis, there being no change in clearance during urea diuresis.

The increase in chloride clearance due to dilution is about the same as that in pressure diuresis in the anaesthetized dog, whereas in the isolated perfused organ dilution diuresis resembles urea diuresis more closely in this respect.

The evidence with regard to the mechanism of dilution diuresis in the anaesthetized dog is, therefore, more definite than it is in the isolated kidney. A twofold increase in urine flow accompanied by little increase in creatinine clearance, which is substantially less than the increase produced in pressure diuresis of the same extent, cannot be attributed solely to an increased rate of glomerular filtration. It is clear that in this preparation dilution of the plasma produces a specific change in tubular activity inhibiting reabsorption of water, and that dilution diuresis is in large part due to this action on the tubules, and only in a smaller degree to an increase in glomerular filtration.

*The comparison of dilution diuresis with glomerular  
and tubular diuresis under isorrheic conditions*

*Isolated kidney.* A more exact analysis of the effects of dilution of the serum can be based on observations in which the increase in urine flow is just prevented by suitable reduction of the pressure head governing the



constant by variations in ureter pressure (Fig. 6); this provides for a direct comparison of the forms of diuresis due to arterial pressure, dilution and urea acting under isorrheic conditions, and probably enables the variations in pressure in the glomerular capsule to be measured fairly accurately [Winton, 1931*a*]; nevertheless we have recently discovered a small effect of ureter pressure on tubular activity [Eggleton, Pappenheimer & Winton, 1940*b*] and this complicates the interpretation of the changes in composition of the urine.

The procedure adopted in experiments designed to compare dilution and pressure diuresis under isorrheic conditions is illustrated in Fig. 5. When the kidney is transferred to the circuit containing diluted blood, the arterial pressure is also reduced to an extent necessitated by the condition that the urine flow is to remain unchanged. This adjustment is not easy and requires experience and constant attention throughout the experiment. The adjustments were made relatively quickly and precisely with the aid of the recording urine flow-meter described by one of us [Winton, 1939]. After a suitable delay during which a steady state was obtained, two or three consecutive samples of urine secreted from the diluted blood were collected. The kidney was then restored to the control circuit, the arterial pressure being raised just far enough to compensate for the more concentrated blood, and after suitable delay two or three successive samples of urine were collected. In this way the composition of the urine and the arterial pressure during isorrheic dilution diuresis could be fairly compared with the average value for the same variables obtained before and after on undiluted blood, in spite of the gradual decline in the creatinine clearance.

The main points demonstrated by Fig. 5 are the order of accuracy of our technique in keeping the urine flow constant, the fact that the steady decline in creatinine clearance is not substantially accelerated or retarded during the periods of dilution diuresis, and the relation between the degree of dilution of the serum and the change in arterial pressure needed to neutralize its effect on the urine flow. For simplicity, the variations in urea and chloride clearances have been omitted from Fig. 5, but included in the summary of data in Table IV.

A corresponding experiment in which an increase in ureter pressure was adjusted so as to neutralize the increase in urine flow due to serum dilution is illustrated in Fig. 6. It shows again the order of accuracy achieved in matching the urine flows, it shows the relation between the equivalent ureter pressures and serum dilution when the serum is progressively diluted, but it differs from Fig. 5 especially in showing a small

systematic reduction in creatinine clearance during dilution diuresis. We have found a similar decline in creatinine clearance when a rise in ureter pressure is neutralized by an increase in arterial pressure, the composition of the blood remaining unchanged [Eggleton *et al.* 1940b], and the decline shown in Fig. 6 cannot, therefore, be attributed to serum dilution.

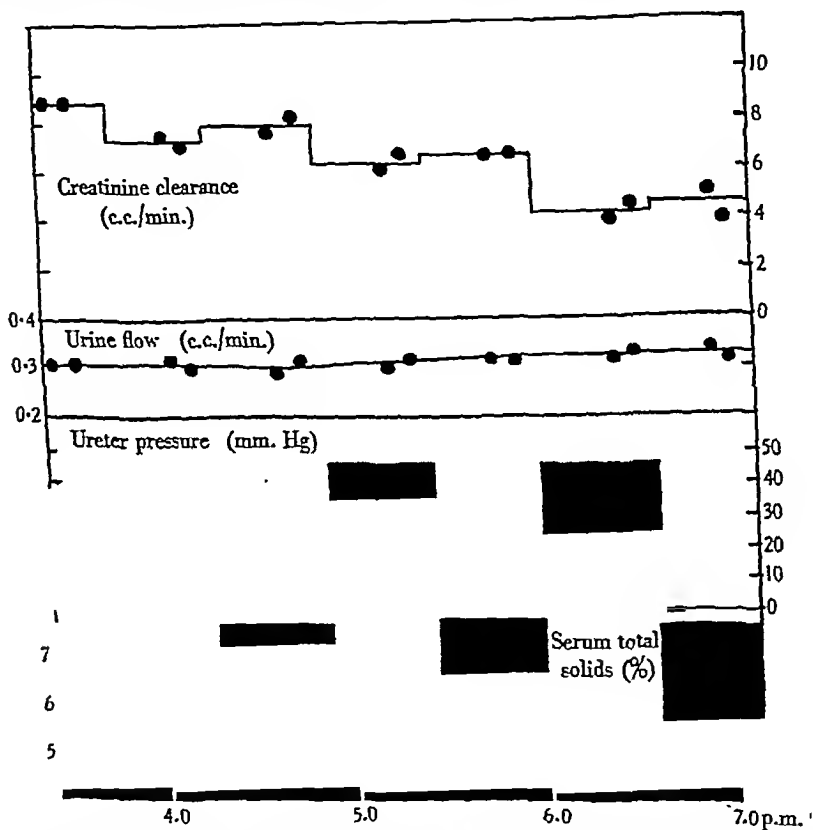


Fig. 6. Isorrheic dilution diuresis in the isolated kidney. Showing relation between ureter pressure and degree of dilution when urine flow is kept constant, and the small decrease in creatinine clearance due to rise in ureter pressure. Double pump-lung single kidney preparation. Kidney weight 20 g. Initial blood flow 135 c.c./min. Arterial pressure 130-132 mm. Hg.

Our observations on isorrheic diuresis in the isolated kidney are summarized in Table IV, all values being reduced to the percentage change per 100 mm. Hg, on the assumption that the change in value is proportional to the change in pressure. This assumption is made for simplicity in presentation, and is only approximately true. It should be

TABLE IV. Isolated kidney. Isorrheic diuresis. Showing the percentage changes in clearances when increase of urine flow due to various diuretic agents was prevented by decrease in arterial pressure or by increase in ureter pressure. Results expressed per 100 mm. Hg change in pressure

Diuretic agent ...	Controlled by changes in arterial pressure Ringer's fluid	Controlled by changes in ureter pressure		
		Arterial pressure	Ringer's fluid	Urea
Percentage change in				
Creatinine clearance	-13.5 ± 15	-47 ± 12	-73 ± 22	-148 ± 15
Urea clearance	+6.6 ± 8	-20 ± 10	-17 ± 20	-144 ± 23
Chloride clearance	-80 ± 39	-19 ± 50	-127 ± 53	-24 ± 52
Number of observations	9	12	11	4
Mean increase in pressure (mm. Hg)	34	45	38	40

noted that whereas 100 mm. Hg change in ureter pressure probably represents nearly the same change in pressure in the glomerular capsule, a change of 100 mm. in arterial pressure probably represents only 60-65 mm. change in the glomerular capillaries. The salient features of these results are (1) with reference to the creatinine clearance, both arterial and ureter pressure experiments indicate an approach of dilution diuresis to pressure diuresis, but taken together both suggest that this approach may be incomplete and that a small inhibition of tubular reabsorption of water may contribute to the phenomena, (2) with reference to urea clearance, the approach of dilution to pressure diuresis seems complete in both types of experiment, urea diuresis giving widely different values, and (3) with reference to chloride clearance, there appears in both types of experiment to be a smaller output of chloride during dilution diuresis than in pressure diuresis, and the few observations on isorrheic urea diuresis suggest that dilution diuresis involves also a smaller output of chloride than does urea diuresis. The averages of the increases in pressure actually observed are given at the foot of the table, and show that the magnitude of isorrheic diuresis was of the same order in the different classes of experiment tabulated.

*Anaesthetized dog.* The observations on the kidney *in situ* secreting under isorrheic conditions were planned less elaborately than those on the isolated kidney for reasons described at the beginning of the section on isobaric diuresis in the whole animal. Many of the experiments summarized in Table V were simpler and more direct in design than that depicted in Fig. 7, which was chosen for illustration because it showed in one experiment many of the important features of both dilution and urea diuresis as manifested in both its isobaric and isorrheic forms. Isobaric

dilution diuresis is shown to produce only a trivial increase in creatinine clearance, but a more considerable increase in the urea and chloride clearance, but a more considerable increase in the urea and chloride

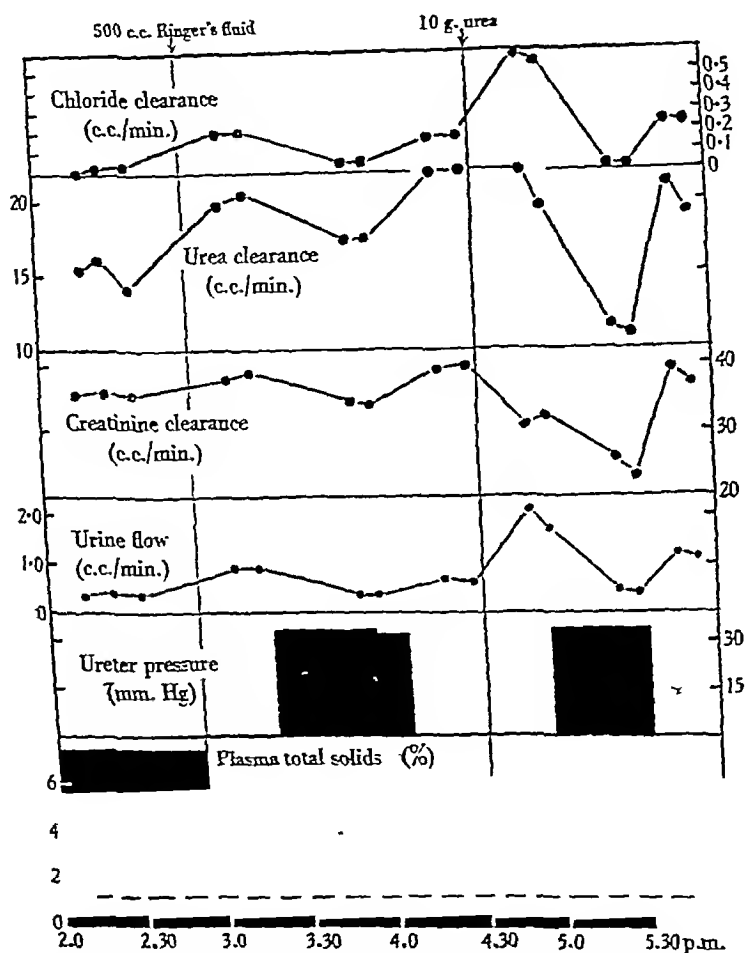


Fig. 7. Isorrheic diuresis in the chloralosed dog. Changes in creatinine, urea, and chloride clearances when the increased urine flow due to (a) Ringer's fluid, (b) urea, is reduced to the pre-diuretic level by rise in ureter pressure. A broken line is inserted at 1% total solids, so that the values above this indicate the approximate concentration of plasma protein. Dog 18 kg. Kidney denervated.

clearances. A rather severe isobaric urea diuresis, on the other hand, is accompanied by a fall in creatinine clearance, no great change in urea clearance and a rise in chloride clearance.

The influence of isorrheic dilution diuresis on the composition of the urine is shown in the summary of our observations in Table V. The chief reason for the differences in the decline of creatinine and urea clearances shown under the headings "arterial pressure" and "ureter pressure" is

TABLE V. Anaesthetized dog. Isorrheic diuresis. Showing the percentage changes in clearances in dilution diuresis when urine flow is prevented from rising by a decrease in arterial pressure or by an increase in ureter pressure. Results expressed per 100 mm. Hg change in pressure

	Controlled by changes in arterial pressure	Controlled by changes in ureter pressure
Creatinine clearance	-57 ± 17	-75 ± 14
Urea clearance	-32 ± 21	-75 ± 20
Chloride clearance	-44 ± 82	+465 ± 245
Number of observations	5	8
Mean increase in pressure (mm. Hg)	56	32

that 100 mm. change in arterial pressure represents only about two-thirds of the change in glomerular pressure which is produced by 100 mm. change in ureter pressure. It is not known whether ureter pressure modifies tubular activity in the whole animal in any way comparable with the small effect of this kind it produces in the isolated kidney. It is clear that if dilution of the plasma is accompanied by a change in pressure, such that there is no change in urine flow, there is reduction in creatinine clearance, and probably also in urea clearance. The changes in chloride clearance are variable.

*The hydrostatic pressure equivalent to a change in the  
colloid osmotic pressure of the serum*

The technique of measuring pressure changes during isorrheic dilution diuresis is sufficiently described in connexion with the experiments charted in Figs. 5-7. These measurements are summarized in Table VI, and for the sake of comparison are expressed as the change in pressure calculated for a change by 1% in plasma protein. The hydrostatic pressures given in the table are thus equivalent in their diuretic effect to a change in colloid osmotic pressure of the plasma of about 3 mm. Hg.

The disproportion between these pressure changes and the change in colloid osmotic pressure is immediately apparent, and corresponds with the disproportion in the extent of the isobaric diuresis due to dilution described above (Table I). The disproportion in the pressure equivalents in comparisons made on the anaesthetized dog might be attributed to the change in tubular activity accompanying dilution diuresis which seemed

TABLE VI. Pressure changes (mm. Hg) required to neutralize the diuretic action of a 1% reduction (e.g. from 8% to 7%) of plasma proteins due to dilution with Ringer's fluid

	Ureter pressure		Anaesthetized dog	
	Isolated kidney	Denervated	Innervated	
Pressure increase	21 $\pm$ 2.1	21 $\pm$ 4	36 $\pm$ 12	
Number of observations	11	3	5	
Average reduction in % concentration of plasma proteins	1.7	1.59	1.34	
	Arterial pressure		Anaesthetized dog	
	Isolated kidney	Denervated	Innervated	
Pressure decrease	15 $\pm$ 1.9	18 $\pm$ 0.3	50 $\pm$ 3	
Number of observations	15	2	3	
Average reduction in % concentration of plasma proteins	2.06	2.23	1.5	

to be indicated by observations summarized in Tables III and V. In the isolated kidney, however, the observations summarized in Tables II and IV have emphasized that an increase in glomerular filtration appears to be the major factor in dilution diuresis.

To appreciate the significance of the values of the pressure equivalents given in Table VI it is essential to take account of the presence of intrarenal pressure and the fact that it appears to increase during dilution diuresis but not during pressure diuresis [Winton, 1936]. Take, for example, the arterial pressure equivalent in the isolated kidney; the 15 mm. change in the artery would correspond with about 10 mm. change in the glomerular capillaries, but to this reduction in pressure head should be added a value, probably about 10 mm., corresponding to the increase in intrarenal pressure due to dilution of the serum, this 10 mm. acting like a back pressure comparable with that of ureter pressure. If there had been no change in intrarenal pressure, the arterial pressure equivalent might well have been 30 mm. instead of 15 mm. In our observations on the ureter pressure equivalent on the isolated kidney we contrived to prevent such masking of the true pressure equivalents by raising the ureter pressure at the beginning of the experiment above any likely intrarenal pressure, and measuring the further increase in ureter pressure equivalent to serum dilution from this base line. This value of 21 mm. may be a few per cent too high for reasons given elsewhere [Eggleton *et al.* 1940b], and our fairest estimate of the change in hydrostatic pressure head across the glomerular membrane equivalent to a change by 1% in serum protein would be 20 mm. Hg in the isolated kidney. The observations on the ureter pressure equivalent in the anaesthetized dog were unfortunately conducted without the precaution of an initial rise in ureter pressure. Since a pre-existing intrarenal pressure would act like a small ureter pressure, whereas when the ureter pressure was raised during isorrheic dilution diuresis this would mask all effects of intrarenal pressure, the values for ureter pressure equivalents may be too high by an amount equal to the intrarenal pressure, if any, in the anaesthetized dog before administration of the diuretic.

Consequently, referring to the values in Table VI, the arterial pressure equivalents are lower than and possibly only one-half the true equivalents, the ureter pressure equivalent on the anaesthetized dog is, if anything, too high, and only the ureter pressure equivalent in the isolated kidney should be accepted as fairly representing the pressure change across the glomerular membrane.



The question as to whether the hydrostatic pressure equivalent is proportional to serum dilution has not been investigated, but the average values of the dilutions actually used were sufficiently alike, as shown in Table VI, in the different classes of our experiments to yield fairly comparable results.

*The change in blood flow through the kidney accompanying dilution diuresis*

It has previously been observed [Whittaker & Winton, 1933] that when an isolated kidney perfused with defibrinated blood is suddenly transferred to perfusion with Ringer's solution at the same temperature, after

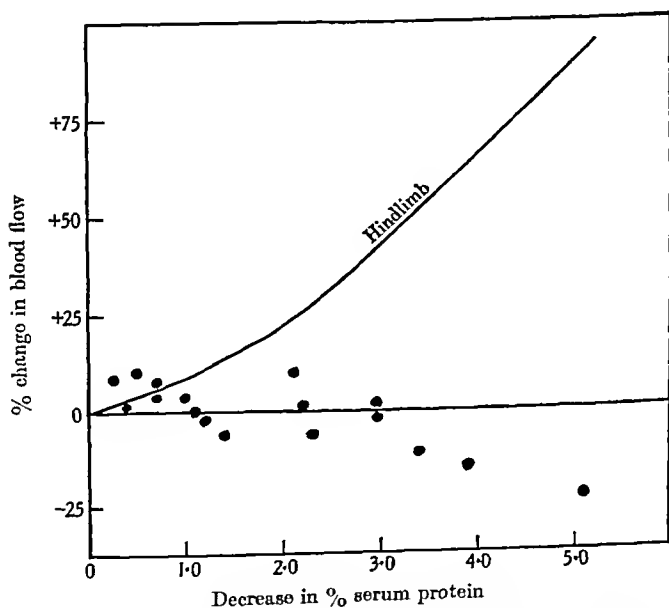


Fig. 8. The percentage change in blood flow through the isolated kidney during dilution diuresis, compared with changes in the hindlimb under similar perfusion conditions.

a quite transient increase, the Ringer flow settles down to a lower value than the blood flow, and is often as low as one-third its original value. This was attributed [Winton, 1937] to the phenomenal increase in intrarenal pressure which obstructs the outflow of the Ringer's solution from the kidney, and so reduces the effective pressure head, thus masking the consequences of reducing the viscosity of the perfusion fluid from that of blood to that of Ringer's solution.

We have recorded the variations in blood flow in all our experiments on isolated kidneys, and though the effects of dilution of the serum are somewhat variable, it would be fair to say that we usually found very little change in blood flow with moderate dilution, and some reduction in flow with considerable dilution. The characteristic relation is well shown in Fig. 8, in which the change in blood flow through the isolated kidney is contrasted with that in the hindlimb of the dog perfused under similar conditions. The curve for the hindlimb is deduced from data given by Whittaker and Winton, appropriate allowance being made for the change in concentration of serum protein in the fashion indicated by Trevan [1918]. The interpretation of the relation in Fig. 8 would appear to be that a small dilution of the blood may produce a small increase in blood flow, appropriate to the reduction in the viscosity of the blood. If dilution proceeds beyond this, the development of intrarenal pressure obstructs the outflow of blood and thus produces a reduction of pressure head which about compensates for the further reduction in the viscosity of the blood. Beyond a dilution corresponding with a reduction of serum protein by about 3% (e.g. from 8 to 5%) this compensation breaks down, the intrarenal pressure rising more steeply and effectively reducing the blood flow.

#### DISCUSSION

The disproportionately great effect of serum dilution when the diuresis due to a given change in colloid osmotic pressure is compared with that due to about the same change in hydrostatic pressure across the glomerular membrane has been shown both in the isolated kidney and in the anaesthetized dog. In the isolated kidney the disproportion appears to average about sevenfold, there being good agreement between the measurements made under isobaric and isorrheic conditions. In the anaesthetized dog, while the disproportion appeared about the same or a little less in a few observations under isorrheic conditions, it was greater, amounting to fifteenfold in the more numerous observations on isobaric diuresis. The fact that the disproportion is greater in the organ *in situ* than after isolation is due to the smaller change in urine flow for a given change in arterial pressure; the increase in flow for a given degree of dilution of the plasma is about the same in the denervated kidney as it is in the isolated kidney (Table I). The interpretation of the disproportion appears to be rather different in the two preparations.

In the *isolated kidney*, comparing equal changes in serum colloid osmotic pressure and in hydrostatic pressure in the glomerulus, dilution produces not only a sevenfold increase in urine flow but about a sixfold

increase in creatinine clearance. Moreover, if the increase in urine flow due to dilution be prevented by reducing the arterial pressure, the isorrheic dilution diuresis is accompanied by a hardly perceptible reduction in creatinine clearance. Now Shannon & Winton (1940) have shown that the creatinine clearance is not a safe guide to the glomerular filtration rate in the isolated kidney, particularly at high U/P ratios. In the experiments described in this communication, however, the U/P ratios were below 40, and, therefore, within the range over which creatinine and inulin U/P ratios are identical. It would seem fair, therefore, to adopt the creatinine clearance as at least a qualitative guide to the rate of glomerular filtration. If so, it is clear that the chief reason for the unexpectedly great diuretic action of serum dilution is an unexpectedly great increase in glomerular filtration, supplemented in quite a minor way by a change in tubular activity, involving a smaller reabsorption of water.

A possibility may be envisaged, that the excessive diuresis following dilution of the blood is due to an increase in glomerular capillary pressure, comparable with that produced by caffeine, without change in renal arterial pressure. This possibility can be excluded for two reasons: (1) diuresis due to considerable dilution could often only be explained by a rise of glomerular pressure well above the arterial pressure, and (2) isorrheic diuresis would be accompanied by a reduction in the viscosity of blood in the vasa afferentia and efferentia, which for reasons already indicated [Winton, 1937] would if anything lower the glomerular pressure slightly in relation to the arterial pressure rather than raise it.

The reason for this great increase in rate of glomerular filtration in dilution diuresis when the pressure head across the membrane is unchanged must be either that the surface area of the membrane is greatly enlarged or that the permeability of the membrane is correspondingly increased.

Reasons have been given against supposing that the proportion of active glomeruli varies in the mammalian kidney [White, 1939], and the grounds for regarding this proportion as constant in the isolated kidney under our conditions have also been given [Winton, 1937]. In any case, a considerable increase in the number of active glomeruli would give quite a different value of creatinine clearance from that observed in the isorrheic form of dilution diuresis. Increase in the surface area of individual capillaries in the glomerular tuft would, however, produce the required consequences, but such a change seems in conflict with the histological evidence [Brodie, 1914] that during diuresis it is the intracapsular space rather than the glomerular tuft which increases in size.

The hypothesis of a variation in permeability of the glomerular membrane has fallen into disrepute because it has commonly been used as a cloak for ignorance of the mechanism of diuresis, and entirely without specific reference to the pressure-flow relation across the membrane. Dilution diuresis in the isolated kidney, however, has now been shown to involve a large increase in filtration rate in circumstances involving little change in filtration pressure (isobaric diuresis), whereas a large reduction in filtration pressure is required to prevent the filtration rate changing from its prediuretic value (isorrheic diuresis). This describes a change in pressure-flow relation which may fairly be taken to define an increase in permeability to water of the glomerular membrane.

When ultrafiltration is proceeding across the glomerular membrane at its usual high rate, the layer of serum in immediate contact with the membrane must contain protein in higher concentration than the average protein concentration of the serum in the capillary. Such a highly concentrated layer would presumably present considerable obstruction to ultrafiltration comparable with that encountered in ultrafiltration through artificial membranes. If so, dilution of the serum might produce a reduction in this obstruction and a fall in pressure head greatly in excess of the small theoretical change associated with the change in colloid osmotic pressure. It is not certain, therefore, that the increase in permeability due to dilution of the serum involves a change in the properties of the cells constituting the glomerular membrane.

The variations in the urea clearance during dilution diuresis will be discussed in a future communication devoted to an analysis of the urea and creatinine clearance ratios in various forms of diuresis. The chloride clearance is substantially lower in dilution diuresis, in both its isobaric and isorrheic forms, than in a corresponding pressure diuresis; this is so in spite of the increase in chloride content of the serum which accompanies dilution produced, as it has been in our experiments, by adding Ringer's solution to the blood. It would seem that such dilution has a direct effect on the tubule cells, promoting reabsorption of chloride, but whether this is an effective stimulus is the reduction of colloid osmotic pressure or some slight change in ionic balance in the serum cannot certainly be decided.

In the *anaesthetized dog* the justification for regarding the creatinine clearance as a measure of the rate of glomerular filtration is supported by the identity of the inulin and creatinine clearances at all U/P ratios [Shannon & Winton, 1940]. In isobaric dilution diuresis there may be

increase in creatinine clearance. Moreover, if the increase in urine flow due to dilution be prevented by reducing the arterial pressure, the isorrheic dilution diuresis is accompanied by a hardly perceptible reduction in creatinine clearance. Now Shannon & Winton (1940) have shown that the creatinine clearance is not a safe guide to the glomerular filtration rate in the isolated kidney, particularly at high U/P ratios. In the experiments described in this communication, however, the U/P ratios were below 40, and, therefore, within the range over which creatinine and inulin U/P ratios are identical. It would seem fair, therefore, to adopt the creatinine clearance as at least a qualitative guide to the rate of glomerular filtration. If so, it is clear that the chief reason for the unexpectedly great diuretic action of serum dilution is an unexpectedly great increase in glomerular filtration, supplemented in quite a minor way by a change in tubular activity, involving a smaller reabsorption of water.

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The reason for this great increase in rate of glomerular filtration in dilution diuresis when the pressure head across the membrane is unchanged must be either that the surface area of the membrane is greatly enlarged or that the permeability of the membrane is correspondingly increased.

Reasons have been given against supposing that the proportion of active glomeruli varies in the mammalian kidney [White, 1939], and the grounds for regarding this proportion as constant in the isolated kidney under our conditions have also been given [Winton, 1937]. In any case, a considerable increase in the number of active glomeruli would give quite a different value of creatinine clearance from that observed in the isorrheic form of dilution diuresis. Increase in the surface area of individual capillaries in the glomerular tuft would, however, produce the required consequences, but such a change seems in conflict with the histological evidence [Brodie, 1914] that during diuresis it is the intracapsular space rather than the glomerular tuft which increases in size.

As disillusion has so often followed the most convincing advocacy of renal theories, it may be well, in conclusion, to summarize those of our results which can easily be expressed in a form independent of theoretical beliefs, even the belief that the creatinine clearance is related to the rate of glomerular filtration. We may set out (Table VII) the characteristic

TABLE VII. The difference between pressure and dilution diuresis expressed as a percentage of the difference between pressure and urea diuresis (from data in Tables II-V)

	Isolated kidney		Anaesthetized dog
	Isobaric diuresis	Isorrheic diuresis	Isobaric diuresis
Creatinine clearance	19	26	42
Urea clearance	24	0	42
Chloride clearance	83	—	17
Total osmotic work	12	—	—

changes in the composition of the urine induced during dilution diuresis, compared with the standards of reference we have chosen, namely, pressure diuresis and urea diuresis. Each figure in the table represents the difference between the values for dilution and pressure diuresis in respect of one variable, expressed as a percentage of the corresponding difference between the values for pressure and urea diuresis. Those who believe that pressure diuresis is primarily due to increase in glomerular filtration, or who define "glomerular diuresis" in this way, and that urea diuresis is primarily due to change in activity of the tubules, or who define "tubular diuresis" in this way, would interpret the values in Table VII as giving a guide to the magnitude of the tubular factors operating in dilution diuresis.

### SUMMARY

1. The mechanism of dilution diuresis has been investigated in the anaesthetized dog and pump-lung-kidney preparation by comparison with pressure diuresis and urea diuresis, both under isobaric conditions, i.e. when the pressure head is not changed, and under isorrheic conditions, i.e. when the urine flow is kept constant by suitable adjustments of arterial or ureter pressure.
2. Dilution diuresis (isobaric) due to a given change in colloid osmotic pressure of the plasma is disproportionately greater than pressure diuresis due to an equivalent change in hydrostatic pressure across the glomerular membrane. The disproportion is about sixfold in the isolated kidney and fifteenfold in the anaesthetized dog (Table I).
3. The increase in urine flow due to a reduction in colloid osmotic pressure of the serum of 3 mm. Hg can just be prevented by a mean

of the same magnitude. The creatinine clearance in considerable isobaric urea diuresis falls, presumably owing to the development of intrarenal pressure, though this has not been measured in the anaesthetized animal. It is evident that dilution diuresis in the whole animal involves a much greater change in tubular activity than in the isolated kidney. The inhibition of tubular reabsorption of water is a factor in the diuresis at least as important as, and possibly more important than, the increase in the formation of glomerular filtrate.

This interpretation is confirmed by those observations on isorrheic diuresis in which dilution of the blood was accompanied by a suitable reduction in arterial pressure, there being a substantial fall in creatinine clearance although the urine flow is unchanged. The significance of the observations in which a rise in ureter pressure counteracted the diuretic influence of blood dilution is doubtful, because no information is yet available about the direct effect of ureter pressure on tubular activity in the anaesthetized animal.

It has become usual in recent years to regard the phenomena of water diuresis and of dilution diuresis as entirely distinct, the former being attributed to inhibition of the tubular reabsorption of water, and the latter to an increase in glomerular filtration. As we have now demonstrated that there is an inhibition of tubular reabsorption of water which is a major factor in dilution diuresis, this distinction is no longer so clear cut.

It should be emphasized that this tubular factor is detected by contrasting dilution with pressure diuresis, and the changes in tubule activity concomitant on increased glomerular filtration in pressure diuresis are not concerned in the evaluation of this factor. The question whether the effect of blood dilution on the tubules is due to a change in colloid osmotic pressure, and if so whether this effect is a direct one on the tubule cells or an indirect, involving possibly some hormonal mechanism in the animal, is under investigation by our colleague Dr L. Podhradszky.

The changes in chloride clearance during dilution diuresis are again rather different in the anaesthetized dog from those found in the isolated kidney. In the whole animal there is in isobaric diuresis an increase in chloride clearance nearly as great as that in pressure diuresis, but since this is accompanied by an increase in creatinine clearance which is much smaller than that in pressure diuresis, we may infer an inhibition of reabsorption of chloride as a consequence of blood dilution. A similar inference may be drawn from our observations on isorrheic dilution diuresis in the anaesthetized animal.

## THE INACTIVATION OF ADRENALINE IN VIVO IN MAN

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EXPERIMENTAL work on the sympathetic nervous system has been held up to a considerable extent by our ignorance of the way in which adrenaline is inactivated in the body. In many experiments, for example, in which the effects of adrenaline are measured, the rate of inactivation of adrenaline comes in as an unknown and possibly variable factor affecting the apparent sensitivity of the system to adrenaline. It has frequently been assumed that adrenaline is destroyed in the body by "autoxidation", but the growing evidence that adrenaline may be concerned in the transmission of impulses by the postganglionic sympathetic neurons raises the question of whether there is an enzymic system in the body which inactivates adrenaline in the same way as choline esterase inactivates acetylcholine.

Experiments *in vitro* have shown that adrenaline is readily oxidized by a number of different enzymic systems. These include (a) catechol oxidase [Abderhalden & Guggenheim, 1908], (b) the cytochrome system [Green & Richter, 1937], (c) amine oxidase [Blaschko, Richter & Schlossmann, 1937; Richter, 1937], (d) peroxidase and (e) *pseudophenolases* such as the copper-protein complexes [Bhagvat & Richter, 1938]; but it is not yet known to what extent any of these systems are concerned in the inactivation of adrenaline *in vivo*.

Bacq [1938] has supported the view that adrenaline is inactivated by a catechol oxidase, but Bhagvat & Richter [1938] were unable to find an active catechol oxidase in mammalian tissues. Gaddum & Kwiatkowski [1938] concluded that adrenaline is oxidized *in vivo* by the amine oxidase, since the effects of adrenaline are augmented by ephedrine which inhibits this enzyme, and they have put forward a theory of the action of ephedrine which is based on this view; but Richter & Tingey [1939] have



increase in ureter pressure of  $21 \pm 2$  (11) mm. Hg in experiments on the isolated kidney so designed as to prevent interference by changes in intrarenal pressure. Disproportionately high arterial and ureter pressure equivalents were also found in isorrheic diuresis in the anaesthetized dog (Table VI).

4. Comparisons of changes in creatinine and urea clearances in both the isobaric and isorrheic forms of diuresis due to dilution, pressure and urea show that dilution diuresis in the isolated kidney is mainly due to increase in the rate of glomerular filtration, whereas in the anaesthetized dog a change in tubular activity involving reduction in the reabsorption of water is a major factor (Tables II-V).

5. In spite of the increase in plasma chloride concentration after administration of Ringer's solution, there is an increased reabsorption of chloride in the isolated kidney. In the anaesthetized dog, on the other hand, dilution diuresis involves diminished reabsorption of chloride.

6. The disproportionately increased glomerular filtration rate during dilution diuresis in the isolated kidney is attributed to increased permeability to water of the glomerular membrane, possibly owing to lessening of an obstruction due to protein in the layer in immediate contact with the membrane, the protein being highly concentrated because left behind during rapid ultrafiltration.

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In order to test for the presence of conjugated derivatives the urine was hydrolysed by heating for 1 hr. with 10% sulphuric acid. It was then found to contain a phenolic compound which was readily separated from the urine by adsorption on aluminium hydroxide and identified as corbasil by (a) the green colour reaction with ferric chloride, (b) the red colour fading to yellow given with aqueous iodine in potassium iodide, and (c) the characteristic violet colour, extractable by butyl alcohol, given by treating at pH 5.2 with alcoholic iodine followed by sodium thiosulphate.

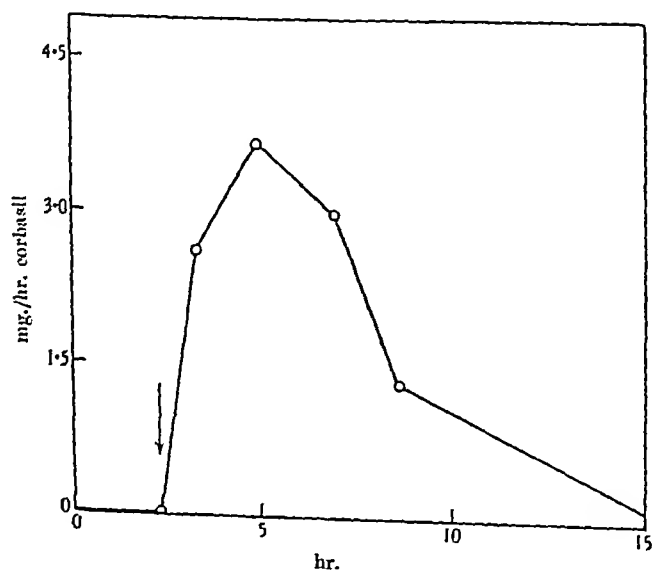


Fig. 1. Rate of elimination of conjugated corbasil derivative after taking 50 mg. at time marked by arrow.

The last colour reaction is due to the formation of an iodoadrenochrome derivative [Richter & Blaschko, 1937] and is very specific for amines of the adrenaline series. The corbasil obtained from the hydrolysed urine gave a brilliant blue-violet colour identical in shade with that given by pure corbasil.

*Rate of elimination.* Urine was collected every 3-4 hr. after taking corbasil (50 mg. base with 0.2 g. glycine and 2 ml. *N*/5 hydrochloric acid to reduce oxidation in the intestine). Conjugated corbasil was estimated in the urine by the method described below for adrenaline. The rate of excretion of conjugated corbasil was maximal from 2 to 6 hr. after administration (Fig. 1) and was still considerable after 9 hr. The total

recently shown that the rate of oxidation of adrenaline by the amine oxidase is probably too slow to account for the rate of inactivation observed *in vivo*. It is therefore impossible to draw any conclusions as yet from these *in vitro* experiments as to the way in which adrenaline is inactivated in the tissues *in vivo*.

A new approach to this problem has now been tried by attempting to identify reaction products in the urine after administering adrenaline and other sympathomimetic amines of the adrenaline series. These amines are all toxic substances which can be given only in relatively small amounts, but some of them are easier to investigate in this way than adrenaline as they can be given in much larger doses.

The inactivation of adrenaline by the amine oxidase might be expected to lead to the formation of protocatechuic acid,  $(\text{OH})_2\text{C}_6\text{H}_3\text{COOH}$ , which would be excreted in conjugated form in the urine. Weinstein & Manning [1937] have reported the excretion of a substance similar to protocatechuic acid, after giving large doses of adrenaline, in the rabbit. Inactivation by catechol oxidase, the cytochrome system or *pseudo*-phenolases forms adrenochrome which is an indol derivative and might be expected to appear in the urine as a conjugated indole derivative.

The experiments with corbasil and other amines were done to obtain further evidence as to the activity *in vivo* of the different inactivating systems.

#### CORBASIL

*dl*-Corbasil,  $(\text{OH})_2\text{C}_6\text{H}_3\cdot\text{CHOH}\cdot\text{CH}(\text{NH}_2)\text{CH}_3$ , which is derived from ephedrine, was chosen for examination in the first place since it is not oxidized by the amine oxidase and inactivation by this enzyme could therefore be excluded. It has previously been shown that amines such as ephedrine, which are not oxidized by the amine oxidase, are generally excreted in the urine unchanged [Richter, 1938], and it was expected that corbasil would also be excreted unchanged unless inactivated by another system.

Preliminary experiments showed that added corbasil could be separated from urine by adsorption on aluminium hydroxide at *pH* 9 and elution with phosphoric acid. It could then be estimated by the iodine method commonly used for estimating adrenaline.

Corbasil is known to be active when taken by mouth [Hartung, Munch, Miller & Crossley, 1931]. Doses up to 50 mg. were taken by the author (76 kg.) by mouth, but no free corbasil could be found in the urine although the methods used were sufficiently sensitive to detect an excretion of 1 mg./hr. or 2%/hr. of the amount taken.

*d*-ADRENALINE

*d*-Adrenaline,  $(\text{OH})_2\text{C}_6\text{H}_3\cdot\text{CHOH}\cdot\text{CH}_2\text{NHCH}_3$ , differs from the natural *l*-adrenaline only in the spatial configuration of the  $\text{CHOH}$  group; it is oxidized by the amine oxidase and cytochrome system but not quite as rapidly as the naturally occurring isomer.

Urine was collected before and after taking 55 mg. *d*-adrenaline (100 mg. *d*-adrenaline ditartrate with 0.2 g. glycine and 1 ml. 2% acetic

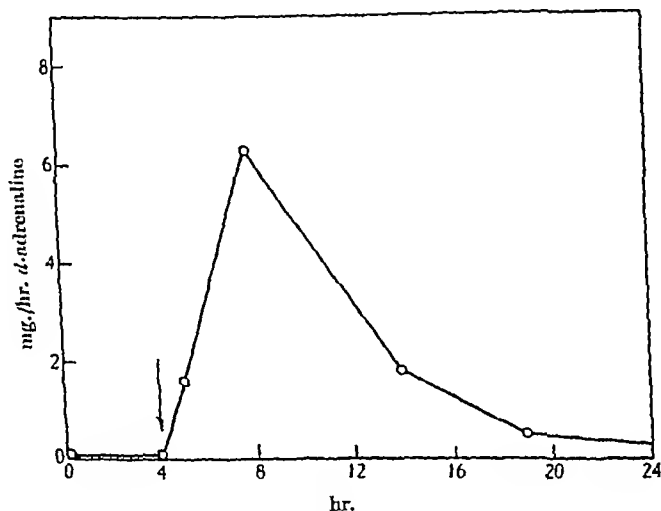


Fig. 2. Rate of elimination of conjugated *d*-adrenaline in urine after taking 55 mg. at time marked by arrow.

acid in 50 ml. by mouth). No free or combined protocatechuic acid and no free *d*-adrenaline was found in the urine, but after hydrolysis with acid *d*-adrenaline could be separated by adsorption on aluminium hydroxide and elution with phosphoric acid; it gave the green ferric chloride reaction and characteristic violet colour of iodoadrenochrome with alcoholic iodine followed by sodium thiosulphate at pH 5.2.

*Rate of elimination.* Conjugated *d*-adrenaline was estimated in the urine by the method given below for *l*-adrenaline. The rate of elimination was maximal some 4 hr. after administration (Fig. 2). The amount of *d*-adrenaline found in the urine after hydrolysis was 39.2 mg. in 21 hr. after administration or 71% of the amount taken.

amount of corbasil found in the hydrolysed urine up to 9 hr. after administration was 24 mg. or 48 % of the amount taken, but the elimination was probably not complete in that time.

### EPININE

Epinine,  $(\text{OH})_2\text{C}_6\text{H}_3\cdot\text{CH}_2\text{CH}_2\text{NHCH}_3$ , is oxidized much more rapidly than adrenaline by the amine oxidase, and might therefore be expected to be oxidized by this enzyme *in vivo* and eliminated as protocatechuic acid, unless conjugation and elimination in the same way as corbasil is a more rapid process. Urine was collected before and after taking 50 mg. epinine by mouth (61 mg. hydrochloride with 0.2 g. glycine and 1 ml. 5 % acetic acid in 50 ml. water). No free epinine was found in the urine on testing by the colour reaction with iodine.

*Test for protocatechuic acid.* The specimens of urine (40 ml.) were heated with 3 ml. conc.  $\text{H}_2\text{SO}_4$  on the boiling water-bath for 30 min., cooled and extracted with 150 ml. ether. The ether was now extracted with 20 ml. 5 % sodium bicarbonate, the extract shaken with an excess of solid sulphanilic acid and treated with 0.1 ml. *N*/10 iodine solution in 5 % potassium iodide. Under these conditions protocatechuic acid gives a red colour visible at concentrations down to 0.02 mg./ml. The tests on the urine were all negative.

*Test for conjugated epinine.* The urine was treated with an excess of 25 % lead acetate and filtered. The filtrate was brought to pH 8 with 5 % ammonia solution (cresol red), allowed to stand for 1 hr. and the precipitate collected by decanting the clear solution and centrifuging the residue. The precipitate was decomposed with a slight excess of *N* sulphuric acid (acid to congo red) and the lead sulphate centrifuged off. The resulting solution, after hydrolysis under the conditions described, gave the characteristic reactions of epinine with ferric chloride and aqueous and alcoholic iodine.

The colour given by epinine with iodine is an orange red which is not suitable for quantitative estimation in the presence of the urinary pigments, but a rough colorimetric estimation indicated that the amount of conjugated epinine in the urine was between 30-60 % of the amount taken.

The green colour given by epinine with ferric chloride depends on the presence of the catechol group. Since this reaction was positive after, but negative before, hydrolysis, the conjugated derivative could not contain a free catechol group; this means that conjugation must occur at one of the phenolic hydroxyl groups.

The normal dose of adrenaline for subcutaneous injection in man is about 1 mg. Doses of 15 mg. adrenaline taken with 0.1 g. glycine and 10 ml. 1% acetic acid in 50 ml. water by the author by mouth 4 hr. after a meal caused marked blanching of the skin, sweating on the face, diarrhoea, a feeling of abdominal discomfort, a rise of 20 mm. in the systolic blood pressure and a rise of 51 mg./100 ml. in the blood sugar (Fig. 3). A dose of 30 mg. adrenaline taken with 0.1 g. glycine and 10 ml. 1% acetic acid in 50 ml. water caused, in addition to these effects, marked glycosuria and severe abdominal pains which persisted for several hours. It was therefore evident that adrenaline was active when taken by mouth when precautions were taken to prevent oxidation in the intestine.

*Elimination.* Analogy with corbasil, epinine and *d*-adrenaline suggested that *l*-adrenaline might also be eliminated by conjugation, though in view of its physiological significance it was possible that there might be an entirely different system specially designed for the inactivation of *l*-adrenaline. Examination of the urine after taking 10–30 mg. adrenaline failed to show the presence of any free adrenaline, but after hydrolysing with acid the presence of adrenaline was shown by every test that was applied. The isolation of small amounts of adrenaline from relatively large amounts of urine presents a technical problem that has not yet been solved, but fortunately adrenaline gives a number of very specific colour reactions which enable it to be identified with a high degree of certainty without actual isolation. The tests applied were best shown after partial purification of the conjugated adrenaline derivative by lead acetate precipitation and extraction with alcohol as described below: the adrenaline derivative was then hydrolysed by heating for 30 min. with 10% sulphuric acid. The tests include (a) the green colour reaction with ferric chloride, due to the catechol nucleus, (b) the red colour fading to yellow with aqueous iodine in KI followed by sodium thiosulphate, due to the formation of adrenochrome, (c) the orange colour given on treating adrenochrome with hydroxylamine, due to the formation of adrenochrome oxime [Green & Richter, 1937], (d) the violet colour on warming adrenochrome with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde), due to the indole structure, (e) the violet colour with alcoholic iodine at pH 5.2 followed by sodium thiosulphate, due to iodoadrenochrome [Richter & Blaschko, 1937], (f) the reduction of arsenomolybdic acid and specific test with NaOH described by Shaw [1938], and (g) the inhibition of isolated strips of rabbit intestine. Tests for free or conjugated proto-catechuic acid or for an increased elimination of indole derivatives, using Ehrlich's reagent, were negative. It was concluded that adrenaline was

*l*-ADRENALINE

*Activity of adrenaline when taken by mouth.* It is commonly stated in the literature that adrenaline is inactive when given by mouth [Gunn, 1939; Martindale, 1936; Tuohy & Essex, 1937], though it has been shown repeatedly that the effects of adrenaline are obtained when it is given by mouth in animals [Menninger, 1927; Dorlencourt, Trias & Paychère, 1922; Giragossintz & Mackler, 1929]. It is to be expected that adrenaline

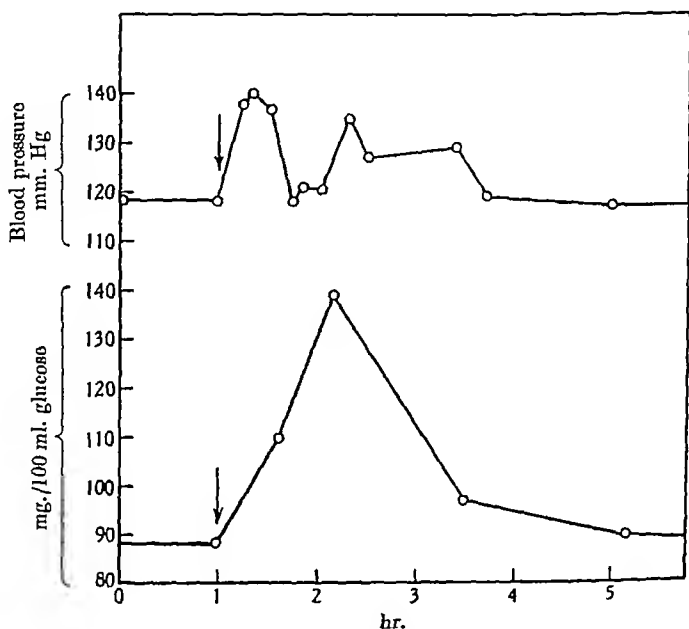


Fig. 3. Systolic blood pressure and blood sugar (by Hagedorn & Jensen method) after taking 15 mg. *l*-adrenaline by mouth at time marked by arrows.

will be partially oxidized at the alkaline *pH* of the intestine unless precautions are taken to prevent this, but the most probable reason for the apparent discrepancy in the literature is that when adrenaline is given by mouth it is considerably diluted and in part inactivated during the passage through the liver before it reaches the general circulation; much larger doses are therefore required to produce a given effect than when it is given subcutaneously or intravenously. In agreement with this view it has been shown that the effects produced by adrenaline are much less when it is injected into the portal vein than when injected into the peripheral veins [Markowitz & Mann, 1929].





eliminated in the same way as the other dihydroxyphenylethylamine derivatives, by conjugation.

*Hydrolysis of conjugated adrenaline.* In order to determine the optimum conditions for hydrolysis flasks containing 40 ml. urine collected after taking 15 mg. adrenaline were heated with 3 ml. conc.  $\text{H}_2\text{SO}_4$  for varying periods on a boiling water-bath and the amount of free adrenaline formed was estimated by the method described below. The optimum time of hydrolysis was about 30 min. and there was a marked falling off in the amount of adrenaline found with longer periods (Fig. 4); this indicated that the hydrolysis was not entirely quantitative, but the amount of adrenaline lost in 30 min. was probably not large.

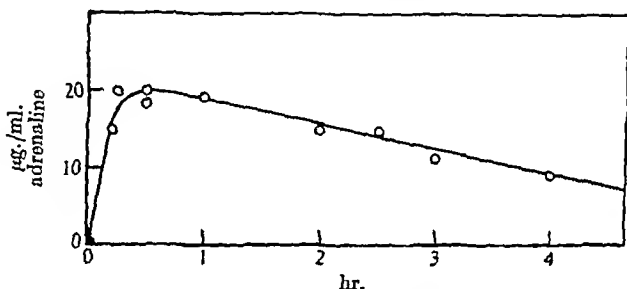


Fig. 4. 1-Adrenaline estimated in urine containing conjugated derivative after hydrolysing for different times.

*Estimation of conjugated adrenaline.* Attempts to estimate adrenaline in the urine by the arsenomolybdate method of Shaw [1938] were only partially successful since it gave a high blank value which had to be subtracted from the figures obtained with adrenaline. The following method, which is similar to that described by Richter & Tingey [1940], was found to be more satisfactory and was generally used:

The urine was hydrolysed by heating 40 ml. with 3 ml. conc.  $\text{H}_2\text{SO}_4$  in a 50 ml. measuring flask for 30 min. on a boiling water-bath. To 15 ml. of the hydrolysed urine was added 0.5 ml. 1.5% glycine, 0.2 ml. saturated sodium acetate and sufficient thymol blue to give a clear red colour. The mixture was cooled by shaking in a wide test-tube in a basin of cold water and brought to pH 4-6 by dropping in 40% sodium hydroxide, care being taken to avoid an excess. A precipitate which formed was filtered off at once and the residue on the filter-paper washed through with 10 ml. water. An aluminium hydroxide suspension (2 ml.) was now added, the mixture brought to a grey-blue colour with 8% sodium hydroxide, centrifuged and the supernatant solution discarded. (The aluminium hydroxide suspension was prepared by adding 10 g. sodium hydroxide in 40 ml. water to a solution prepared by dissolving 50 g. potassium alum in 400 ml. hot water and cooling to about 20°; the precipitate was filtered, washed twice with water and suspended in 200 ml. water.) The precipitate containing adsorbed adrenaline was shaken with 1 ml. 25% sodium dihydrogen phosphate and 6 ml. water. The solution obtained on centrifuging again was decanted into a



TABLE I. Recovery of amines in the urine

Exp.		Dose mg. base/ 76 kg.	Time urine was collected hr.	Amount found in urine mg.	Percentage recovery
1	Corbasil	50	9	24	48
2	Epinine	50	15	15-30	30-60
3	d-Adrenaline	55	21	30-2	71
4	l-Adrenaline	10	7	3-6	30-60
5	"	10	20	5-5	55
6	"	15	9	10-5	70
7	"	30	20	20-7	60

(Estimations by the iodine method except in Exp. 5 in which the arsenomolybdate method was used.)

The concentration of conjugated adrenaline in the urine rose to a value corresponding to  $33\mu\text{g.}$  adrenaline/ml. in the urine obtained 5 hr. after taking 30 mg. adrenaline. Taking the blood volume as 5 l., the concentration in the blood could not be higher than  $6\mu\text{g./ml.}$  so that the kidneys must be able to concentrate the derivative to a considerable extent.

*Purification of conjugated adrenaline derivative.* Attempts to isolate the adrenaline derivative from the urine have not hitherto succeeded, but methods have been worked out which enabled a partial purification to be effected. In this work various methods of fractional precipitation, etc., were tried and the behaviour of the adrenaline derivative was followed by hydrolysing the fractions and estimating adrenaline as described.

The adrenaline derivative was precipitated by lead acetate at alkaline but not at neutral pH, and by making use of this it could be separated from a large number of other substances present in the urine. An excess of 25% lead acetate solution was added to the urine, the bulky precipitate filtered off and the filtrate adjusted to pH 9 with ammonia. On standing, a fine precipitate separated. This was collected, washed and decomposed by adding dilute sulphuric acid (strongly acid to congo red). The adrenaline derivative was found in the clear solution obtained on centrifuging.

A further considerable purification was obtained by evaporating the aqueous solution nearly to dryness and extracting with a small volume of 95% alcohol. This dissolved most of the adrenaline derivative and left a gummy residue of impurities. On fractionally precipitating the alcoholic solution with barium hydroxide the adrenaline derivative was present mainly in the later fractions. When the barium compound was decomposed with sulphuric acid and the resulting solution, after neutralizing with sodium hydroxide, was slowly evaporated in a desiccator, various

sodium salts crystallized out and were separated. The adrenaline derivative was then left as a hygroscopic amorphous mass. Many attempts to crystallize a potassium or a barium salt were unsuccessful.

The conjugated adrenaline derivative differed from adrenaline in that it was not adsorbed on aluminium hydroxide at pH 9 and this could be used for separating them. It was very soluble in water, soluble in 90% alcohol, less soluble in absolute alcohol, slightly soluble in ether, insoluble in chloroform. It was decomposed by heating for 30 min. with dilute sodium carbonate.

*Chemical constitution of conjugated derivative.* The conjugated adrenaline did not give the green colour characteristic of the catechol grouping with ferric chloride, though this reaction was readily obtained after hydrolysis. This indicated that conjugation had occurred at one of the phenolic hydroxyl groups.

Analogy with the elimination of other phenols made it appear likely that adrenaline may be eliminated either as a sulphate ester or as a glucuronide. Partially purified specimens of the adrenaline derivative gave negative glucuronide tests with naphthoresorcinol and showed less than 20% hydrolysis after incubation for 5 hr. at 37° with emulsin; tests for a sulphate ester were positive. No final conclusions can be drawn until the conjugated adrenaline derivative has been isolated in the pure state, but these preliminary experiments show that it has the properties of an ester in that it is easily hydrolysed by acids and suggest that it may be adrenaline-3 or 4-*o*-hydrogen sulphate, which would normally exist as a zwitterion,  $\overline{\text{SO}}_3\text{O}(\text{OH})\text{C}_6\text{H}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\overset{+}{\text{N}}\text{HCH}_3$ .

*Elimination of adrenaline in the rabbit.* A large rabbit (2.5 kg.) was given 20 mg. adrenaline with 0.1 g. glycine and 1 ml. 1% acetic acid in 20 ml. water. No free adrenaline was found in the urine, but the presence of conjugated adrenaline was indicated by positive tests for adrenaline after hydrolysis with acid. Weinstein & Manning [1937] reported the excretion of protocatechuic acid after administering adrenaline; but they hydrolysed the urine by heating with alkali. Since protocatechuic acid is formed from adrenaline on heating with alkali, it may have come from adrenaline in the urine. The excretion of protocatechuic acid in the rabbit would therefore appear to require reinvestigation.

## DISCUSSION

The experiments described give evidence that adrenaline administered in doses of 0.13–0.4 mg./kg. in man is mainly eliminated in the urine in the form of a derivative which has the properties of an adrenaline ester in

which esterification has occurred at one of the phenolic hydroxyl groups. This ester has not yet been obtained pure, but preliminary experiments suggest that it may be the sulphate ester and the inactivating system the "sulphosynthase" or system responsible for the conjugation of phenols with sulphate in the body.

The observation that adrenaline is eliminated by conjugation instead of being oxidized by the amine oxidase in the same way as phenylethylamine and most other amines [Richter, 1938], indicates that conjugation is more rapid than oxidation by the amine oxidase and suggests that it is probably the main physiological method by which adrenaline is inactivated in the body. The detoxication of phenols by conjugation is known to occur mainly in the liver [Pelkan & Whipple, 1922] and this agrees with the physiological evidence that the liver is also particularly effective in removing adrenaline from the circulation [Elliott, 1905; Trendelenburg, 1929].

The simplest interpretation of the present observations is that there is no special system in the body for the inactivation of adrenaline, but that it is detoxicated by conjugation in the same way as other toxic phenols. On the other hand, the work of Quick [1932] challenges the view that the "sulphosynthase" and "glycuronidosynthase" are primarily concerned in detoxication and indicates that these systems may have some more important physiological function. It is suggestive that under normal conditions the conjugation of the simple phenols by these systems is generally incomplete and a considerable proportion of the phenol may be eliminated unchanged [Novello, Wolf & Sherwin, 1925], while with adrenaline the conjugation was remarkably complete and no unchanged adrenaline was ever found in the urine. This suggests that the inactivating system may be specially adapted for inactivating adrenaline, which is probably more toxic and of greater physiological significance than the other phenols normally eliminated in this way.

It should be possible to test experimentally the view that adrenaline is normally inactivated by conjugation by administering other phenols, which should inhibit the inactivation by competing with adrenaline for the inactivating system and should so increase and prolong the effects of adrenaline. This experiment has already been carried out. Many years ago Barger & Dale [1910] observed the effect of catechol in raising the blood pressure, and more recently Bacq [1936] has shown with a series of polyphenols that they augment and prolong the effects of adrenaline and of stimulating the sympathetic nerves *in vivo*.

Bacq concluded that the polyphenols act by inhibiting the autoxida-

tion of adrenaline since phenols have been shown to act as autoxidation inhibitors *in vitro*. This interpretation is very improbable since the tissues already contain autoxidation inhibitors which are far more powerful than the relatively small amounts of phenols administered: there is also no evidence that adrenaline is oxidized *in vivo*. The present work suggests that adrenaline is mainly inactivated by conjugation and that the polyphenols act in augmenting and prolonging the effects of adrenaline by inhibiting this process. In so far as it is possible to draw an analogy between the adrenergic and cholinergic mechanisms, the parts played by eserine and choline esterase would appear to be represented in the adrenergic system by the polyphenols and "sulphosynthase".

### SUMMARY

1. Corbasil, epinine and *d*- and *l*-adrenaline administered in doses of 0.13-0.66 mg./kg. by mouth in man are mainly eliminated in the form of conjugated derivatives in the urine.
2. Conjugation occurs on one of the phenolic hydroxyl groups and the derivatives formed are probably the sulphate esters.
3. The rates of elimination of conjugated corbasil and *d*- and *l*-adrenaline have been measured.
4. Up to 70% of the amounts of adrenaline administered were found in the urine after hydrolysis.
5. Adrenaline is active when given by mouth if precautions are taken to prevent oxidation in the gut.
6. Epinine and *d*- and *l*-adrenaline are not eliminated to any measurable extent as protocatechuic acid.
7. It is concluded that conjugation is the main physiological process by which adrenaline is inactivated in the body and that the inactivation of adrenaline in this way may be intimately related to the observed activity of adrenaline and the functioning of the sympathetic nervous system *in vivo*.

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THE EFFECT OF RIBOFLAVIN, RIBOFLAVIN-  
PHOSPHORIC ACID AND THE CORTICAL  
HORMONE ON THE SURVIVAL OF ADRENAL-  
ECTOMIZED RATS RECEIVING NORMAL AND  
RIBOFLAVIN-DEFICIENT DIETS

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VERZÁR & LASZT [1936] have put forward the very interesting theory that one of the functions of the adrenal cortex is to facilitate phosphorylation in the animal body, and that during adrenal cortical insufficiency the phosphorylating capacity of the organism is impaired. The conversion of riboflavin to riboflavinphosphate is, therefore, according to this theory, one of the metabolic processes which is disturbed by a lack of the cortical hormone.

Riboflavin is pre-eminently a growth factor. If it is withheld from the diet of young rats growth ceases after a few weeks, and will only take place again when riboflavin or riboflavinphosphate is supplied. For intact rats there is no apparent difference between the two; riboflavin is converted to the phosphate and the young rat can use the vitamin in either form. After adrenalectomy however, according to Verzár & Laszt, this is no longer possible and the vitamin must be supplied in the phosphorylated form. This supposition seemed to us to offer a starting point from which an improved method of assay of extracts of the adrenal cortex might be evolved. The sensitivity of the test could be increased by adding riboflavin to the diet, and the survival time of the controls could be decreased by removing riboflavinphosphate from the diet. It was hoped that the resulting graded growth response could be used to interpret adrenal cortical activity with a greater degree of accuracy than has hitherto been possible. With this end in view an examination of the phosphorylation theory as applied to the conversion of riboflavin to riboflavinphosphate was undertaken.



## EXPERIMENTAL

The work reported in this paper can be separated into the following groups:

(1) Experiments to show the effect of riboflavin, riboflavinphosphoric acid and cortical extract on the survival time after adrenalectomy of young rats receiving a riboflavin-free diet (a) whose stores of this vitamin were being depleted during the course of the experiment, (b) whose stores of riboflavin had been fully depleted before the start of the experimental period.

(2) Similar experiments to show the effect of these substances when the diet was complete, for rats from two different colonies.

(3) Experiments to show the influence of the particular diets themselves on the survival time after adrenalectomy.

*Operative technique.* Young albino rats of either sex of 35–45 g. are employed. The rat is anaesthetized with nembutal (pentobarbital sodium), 0.15–0.20 ml. of a solution containing 0.65 mg./ml. being injected intraperitoneally. Anaesthesia develops in about 5 min. and is deepest after about 20 min. The anaesthesia may be completed by the judicious administration of small amounts of ether. We have found this method of anaesthetization very suitable for this operation. Since the rats did not fully recover from the anaesthetic for some hours after the operation, they were given a few drops of a 1 in 4 sugar solution by the mouth, when they were sufficiently recovered to take it, in order to minimize any effect due to partial starvation overnight. The operation was performed as described by Schultzer [1936]. Of 1044 rats operated in this way, in these and other experiments, 114 (10.9%) failed to survive 48 hr. after the operation, and 42 (4.0%) failed to die after cessation of treatment. Of these 42 rats, remnants of cortical tissue were found on post-mortem on the right side in all except two. These figures are similar to those reported by other workers [Schultzer, 1936; Blanchard & Tallman, 1938].

*Housing and care of rats.* The rats used in these experiments were albinos from an inbred colony from the Nutrition Department of the Pharmaceutical Society, except in Exps. 10 and 13 in which black and white rats were used from an inbred colony, and were kindly supplied by Dr S. K. Kon at the National Institute for Research in Dairying. The rats were obtained at weaning, which was about 23 days, and their weights were between 35 and 45 g. They were housed in cages with wire-mesh grid bottoms, and wood-wool was supplied as bedding. Fresh tap

water was given each day. In the first three experiments each group of rats was placed in one cage, but in subsequent experiments each animal was put in a separate cage, since there was reason to believe that the eating of a dead animal by the other animals decreased the survival time of the latter. Litters and sexes were divided as equally as possible among the various groups in any one experiment. The animals were weighed each day to within 1 g.

*Weight.* Change in body weight has been calculated at death for those animals which failed to survive the test period, and at the cessation of treatment for those rats which did not die until after the treatment had been stopped.

Riboflavin deficiency first retards and then prevents growth, which is resumed only when the dietary deficiency is made good. Adrenalectomy also inhibits growth in normal animals, and as all our animals were adrenalectomized we found it necessary to give adrenal cortical hormone in order to induce growth. Those animals which suffered from riboflavin deficiency as well as from adrenalectomy did not grow unless both adrenal cortical hormone and riboflavin were given (Tables I and II). In Exps. 1d and 2d even this treatment failed to cause an increase in weight, probably because the diet was also lacking in the other members of vitamin B complex.

In no group of our rats did the average daily increase in weight reach the expected value for normal young rats of the same age. This was because the doses of active substances given were so chosen as to give a medium and not a maximum response, in order that weight changes of either a greater or a smaller magnitude could be observed in any one experiment.

*Dosing solutions.* The extract of the cortical hormone, 1 ml. of which was equivalent to 75 g. of adrenal cortex, was a sample of eucortone (Messrs Allen and Hanburys, Ltd.) diluted when necessary in normal saline so that half the daily dose was contained in 0.1 ml., given subcutaneously, morning and afternoon. The riboflavin was prepared from the crystalline material so that 50  $\mu$ g. were contained in 1.0 ml. and was freshly made at least once a week in glass distilled water which was slightly acidified with acetic acid. Except for Exp. 1, where subcutaneous injections were given, the riboflavin sweetened with a drop of syrup was supplied to the rats in small individual dishes. No difficulty was experienced with animals refusing a dose. The riboflavinphosphoric acid was a sample of "Lactoflavinphosphorsäure" in ampoules, 2 ml. containing 1.21 mg. kindly supplied by Dr R. Oddie of Messrs Bayer and

Co., Ltd. (This solution retained its biological activity for several months. Seven rats, which had remained approximately steady in weight for nearly 4 weeks were given a daily dose for 6 days of 30.25  $\mu$ g. riboflavinphosphoric acid which had been stored for 5 months. Growth restarted at once in all cases and the rats grew from 15 to 22 g., average 18.4 g. in the week.) It was given directly into the animals' mouths by means of an Agla micrometer syringe. As adrenalectomized rats appeared to be especially sensitive to external influences of any sort, in the later experiments animals, including controls which were not being treated with any particular active substance were, nevertheless, given an equivalent dose of the medium in which the active substance was being administered to other animals. Thus all rats received an injection, whether of active substances or only saline, and all rats received a dose by mouth, water or weak syrup being given to those rats which did not receive riboflavinphosphoric acid. Yeast was used in some experiments as a rich source of riboflavinphosphate, the same sample of dried brewer's yeast being used throughout, except in Exp. 3 f, where baker's yeast was used.

Treatment was started 24 hr. after operation, and those rats which failed to survive a further 24 hr. after the first dose or which failed to die after cessation of treatment and showed remnants of cortical tissue on post-mortem examination were discarded as unsuitable.

*Counting of survival times.* Survival times were counted from the time of operation itself, so that animals received the first treatment on day 1. Counts were made each day first thing in the morning. Animals which survived until the morning but died during the day were counted as having survived until the next day.

*Interpretation of the results.* The variation among members of any one group was considerable. The variance,  $\sigma^2 = \frac{\sum d^2}{(n-1)}$ , where  $d$  = the difference of each individual result from the mean for that group and  $n$  = the number of animals in the group, and the standard error  $\epsilon$ , were calculated for each group of rats. The significance of the difference between the two means was tested by a calculation of the value of " $t$ " from the formula  $t = \frac{m_1 - m_2}{\sqrt{(\epsilon_1^2 + \epsilon_2^2)}}$ , where  $m_1$  and  $m_2$  are the two mean values, and  $\epsilon_1$  and  $\epsilon_2$  the standard errors of those means. From tables of " $t$ " the probability of the observed difference being due to chance was found.

*Experiments with riboflavin deficient diets.* (a) In the first experiments (Table I) the rats received the riboflavin-free diet either on the day following the operation or for some days before, but adrenalectomy was



TABLE I. The effect of riboflavin, riboflavinphosphoric acid, yeast and cortical extract on the survival time after adrenalectomy of young rats receiving a riboflavin-deficient diet

Exp. no.	Group	Treatment (per day)	No. of rats	No. which survived 10 days	Average increase in wt., g.		Average survival time days	Probability that differences are due to chance	
					Until death	During dosing period			
1	a	None	6	1	-6.2	—	9.5	a-b	>0.05
	b	Riboflavin 20 µg.	9	2	-3.8	—	7.7	—	<0.1
	c	Eucortone 0.2 ml.	9	9	—	-2.8	12.4	a-c	>0.001
	d	Riboflavin 20 µg. + eucortone 0.2 ml.	10	10	—	+1.4	12.2	—	<0.01
2	a	None	6	0	-4.8	—	7.2	a-b	>0.6
	b	Riboflavin 10 µg.	6	1	-5.2	—	7.7	a-c	<0.001
	c	Eucortone 0.1 ml.	6	6	—	-3.5	12.7	c-d	>0.2
	d	Riboflavin 10 µg. + eucortone 0.1 ml.	6	6	—	-1.7	13.3	—	<0.3
	e	Dried yeast 5% in the diet	7	1	-3.0	—	8.6	a-e	>0.3
3	a	None	7	5	+0.4	—	9.7	a-c	<0.4
	b	Riboflavin 20 µg.	6	3	+0.3	—	9.3	a-c	>0.2
	c	Riboflavinphosphoric acid 20 µg.	7	2	-3.0	+3.0	7.7	a-d	<0.001
	d	Eucortone 0.2 ml.	7	7	-1.4	+2.7	15.0	b-c	<0.3
	e	Riboflavin 20 µg. + eucortone 0.2 ml.	9	9	+2.0	+5.9	14.1	d-e	>0.2
	f	Baker's yeast 5% in the diet	8	3	-4.0	—	9.6	—	<0.001
	a	None	5	4	-0.8	—	10.0	—	—
4	b	Riboflavin 40 µg.	7	5	+0.4	+3.0	12.9	a-b	>0.1
	c	Riboflavinphosphoric acid 40 µg.	7	4	-0.6	+2.3	9.9	b-c	>0.2
									<0.3

For this whole series of experiments treatment was stopped after 10 days.



TABLE II. The effect of riboflavin, riboflavinphosphoric acid and cortical extract on the survival time after adrenalectomy of young rats whose stores of riboflavin were depleted before the operation

Exp. no.	Group	Treatment (per day)	No. of rats	Average increase in wt., g.		Average survival time, days	Probability that differences are due to chance
				Until death	During dosing period		
5	a	None	8	- 2.8	—	2.9	a-b <0.001
	b	Eucortone 0.1 ml.	9	- 1.9	—	7.4	—
	a	None	9	- 2.7	—	4.3	—
	b	Riboflavin 50 µg.	7	+ 2.4	—	10.6	b-c 0.2
6	c	Eucortone 0.2 ml.	9	- 3.6	—	7.3	a-b >0.001 <0.01
	d	Riboflavin 50 µg. + eucortone 0.2 ml.	6	+ 10.0	+ 29.4 (5 rats)	>29.2	a-c >0.05 <0.1
7	a	None. No operation	6	—	+ 1.0*	>15.0	—
	b	None. Dummy operation	6	—	+ 1.3*	>15.0	—
	c	Riboflavin 25 µg.	9	- 2.7	—	5.1	—
	d	Riboflavinphosphoric acid 30.25 µg. (= 25 µg. riboflavin)	10	+ 1.0	—	>11.5	b-c >0.7 <0.8
	e		11	+ 0.6	—	>12.1	—

\* At 15 days from the start of the experimental period, when the animals were killed.

TABLE III. The effect of riboflavin, riboflavinphosphoric acid, yeast and cortical extract on the survival time after adrenalectomy of young rats receiving a complete diet

Exp. no.	Group	Treatment (per day)	Diet	Strain of rats	No. of rats	Average increase in wt., g.		Average survival time, days	Probability that differences are due to chance
						Until death	During dosing period		
8	a	None	B	Wistar	10	- 0.1	—	5.1	a-b >0.6 <0.7
	b	Riboflavin 20 µg.			8	+ 3.6	—	5.5	a-c >0.6 <0.7
	c	Riboflavinphosphoric acid 20 µg.			10	+ 1.4	—	5.5	a-d <0.001
	d	Eucortone 0.2 ml.			9	+ 15.7	+ 12.8	11.8	—
	e	Dried yeast 1.5% in the diet			10	- 0.8	—	1.4	a-e >0.2 <0.3
9	a	None	B	Wistar	12	+ 2.7	—	7.3	a-b >0.02 <0.05
	b	Dried yeast 1.5% in the diet			12	+ 0.6	—	5.3	—
	c	Dried yeast 10% in the diet = 30 mg intake daily = 170 mg. intake daily			11	- 1.2	—	4.1	a-c <0.001
10	a	None	A	Black and white	12	+ 0.8	—	4.9	—
	b	Riboflavinphosphoric acid 32.5 µg.			8	+ 1.3	—	4.8	b-c >0.05 <0.1
	c	Eucortone 0.01 ml.			9	+ 4.2	—	6.8	a-c >0.05 <0.1
	d	Eucortone 0.05 ml.			9	+ 0.7 (3 rats)	+ 17.2 (6 rats)	8.0	a-d >0.001 <0.01





TABLE IV. The effect of diet on the survival time of young rats after adrenalectomy

Exp. no.	Diet	Strain of rats	No. of rats	No. which survived 10 days	Average survival time days	Probability that differences are due to chance
11	Riboflavin-free + wheat extract	Wistar	8	4	6.9	—
	Stock diet B		9	0	5.4	$>0.3 < 0.4$
12	Riboflavin-free without wheat extract	Wistar	11	10	11.4	$<0.001$
	Stock diet B		10	1	6.0	—
13	Riboflavin-free without wheat extract	Black and white	9	0	6.8	—
	Stock diet A		11	0	3.8	$<0.001$
14	Riboflavin-free without wheat extract	Wistar	6	2	9.5	—
	Stock diet A		6	1	5.8	$>0.1 < 0.2$

The experiment was repeated four times. In three out of four experiments the rats receiving the riboflavin-free diet survived longer and showed a greater variation than their litter mates receiving either of the stock diets, and it must be concluded that a difference exists between these two types of diet. Although in all the three experiments when the diet was riboflavin-free the survival time was increased, and the addition of wheat extract in one experiment appeared to abolish this effect, there is no reason to believe that the absence of riboflavin, or of any of the other members of the vitamin B<sub>2</sub> complex, is solely responsible for the prolongation of the survival time after adrenalectomy. On the contrary, it appears from the experiments recorded in Table II that if the rats themselves are in a state of riboflavin deficiency, they are then more than usually susceptible to lack of cortical hormone. Protein, carbohydrate, sodium chloride and potassium, and calorie value have all been shown to influence the survival time after adrenalectomy [Cleghorn *et al.* 1936]. Without making a detailed analysis of all the possibilities it would be misleading to attribute the difference observed in this case to any particular factor. It is however possible that here the effect is due to the sodium and potassium contents of the diets. On analysis<sup>1</sup> the following values were found in respect of these two elements: riboflavin-free diet: sodium 14.87 mg./g., potassium 1.84 mg./g.; stock diet A: sodium 7.58 mg./g., potassium 5.64 mg./g. Both the higher sodium and the lower potassium intake on the riboflavin-free diet would tend to prolong life after adrenalectomy. These results demonstrate very clearly the necessity of including a negative control group in every experiment

<sup>1</sup> We are indebted to Mr G. B. West of the College of the Pharmaceutical Society for the analysis of these two diets.



the riboflavin made no difference. Exp. 6 gave results more comparable with those of Verzář & Laszt. Although both eucortone and riboflavin prolonged life when given separately the effect was much increased when they were given together. The fact that when rats are suffering from extreme deficiency of riboflavin as well as cortical deficiency, it is necessary to make good both deficiencies in order to prolong life indefinitely, does not show that there is any fundamental connexion between them.

(4) Riboflavinphosphate should differ from riboflavin in that it should prolong life indefinitely, even when no eucortone is given. Verzář & Laszt found that it prolonged life both on a normal diet and on a riboflavin-free diet. When the effects of riboflavin and riboflavinphosphate were directly compared, both on complete and deficient diets in our Exps. 3, 4, 7 and 8, there was no significant difference between them. The small differences which occurred were in the contrary direction to that required by the theory. The doses used were 20–40  $\mu$ g. per day of the pure substances. The result cannot be explained on the assumption that the riboflavinphosphate had dissociated, since it was found to contain no free phosphate after the experiments were completed. Verzář & Laszt used 40–60  $\mu$ g. per day of a preparation containing 33% of riboflavinphosphate. Their result might possibly be due to other substances in their preparation, but if this was so, their result would not support their theory.

(5) Verzář & Laszt found that the addition of yeast to the diet prolonged life and attributed this to the riboflavinphosphate in the yeast. In our Exps. 2, 3 and 8 the addition of yeast, either to complete or to deficient diets, had no significant effect on the survival time, but in Exp. 9 the addition of large quantities of yeast actually shortened life. There is no evidence of what constituent of the yeast had this effect, but it may have been potassium.

The results can be summarized in the statement that we have repeated all the experiments described in the paper by Verzář & Laszt, and have consistently failed to confirm the results on which their theory depends. We believe that the explanation is to be found in the fact that they based all their conclusions on the comparison of the rats used in one experiment with the rats used in another experiment, presumably carried out at a different time. The survival time of adrenalectomized rats is liable to vary from time to time, owing to numerous causes, known and unknown, and false conclusions may be reached unless litter-mate controls are used. If our investigations had been confined to the groups

of rats numbered 2a, 6c, 1b, 7c and 3f they might have been interpreted as confirmation of Verzář & Laszt's theory.

Another possible source of error is the inclusion of rats from which the adrenals have not been completely removed. In our experiments, data obtained from the small number of such rats, which did not die when injections of eucortone ceased, were rejected. There is no evidence that Verzář & Laszt took this precaution, so that some of their long survivals may have been due to this cause.

Although Pijoan & Oberg [1937] confirm the original findings of Verzář & Laszt [1936], their results are not described in detail. Recent interesting results in connexion with the cortical hormone and phosphorylation have been published by Ochoa & Rossiter [1940]. Attempting to confirm Laszt's [1938] suggestions that the cortical hormone is essential for the formation of cocarboxylase (vitamin B<sub>1</sub> pyrophosphate) from vitamin B<sub>1</sub>, they carried out direct determinations of cocarboxylase in adrenalectomized rats. They found no evidence that the cortical hormone has any influence on the phosphorylation of vitamin B<sub>1</sub>.

### SUMMARY

1. The evidence for the theory that extracts of the adrenal cortex prolong the life of adrenalectomized rats by the phosphorylation of riboflavin was not confirmed.

2. When rats lacked both riboflavin and cortical hormone, they eventually died unless both deficiencies were made good, but riboflavin prolonged life in the absence of the cortical hormone, and the cortical hormone prolonged life when there was no riboflavin in the intestine.

3. The effects of riboflavinphosphate on the weight and survival of adrenalectomized rats were the same as those of riboflavin.

4. Yeast did not prolong the life of the rats but shortened it.

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While this paper was in the press we have seen in the literature a paper by Marrazzi & Gaunt, *Proc. Soc. exp. Biol. Med.* (1939), 41, 65, which we had previously overlooked, in which they tested Verzár & Laszt's claim that flavin phosphate, as present in yeast, will prolong the lives of adrenalectomized rats, whereas non-phosphorylated flavin will not. They failed to confirm this since it was found that 59 adrenalectomized rats fed on a high yeast diet (5-10 % of brewer's yeast in the stock diet) had an average survival time of 7.3 days as compared with 8.1 days for 56 control animals which received no extra yeast. Our experiments 8e and 9b and c are in complete agreement with this.



a rectangular strip without any differences in illumination. The collimators were pivoted on axes immediately under the centre of the grating on which they directed the light.

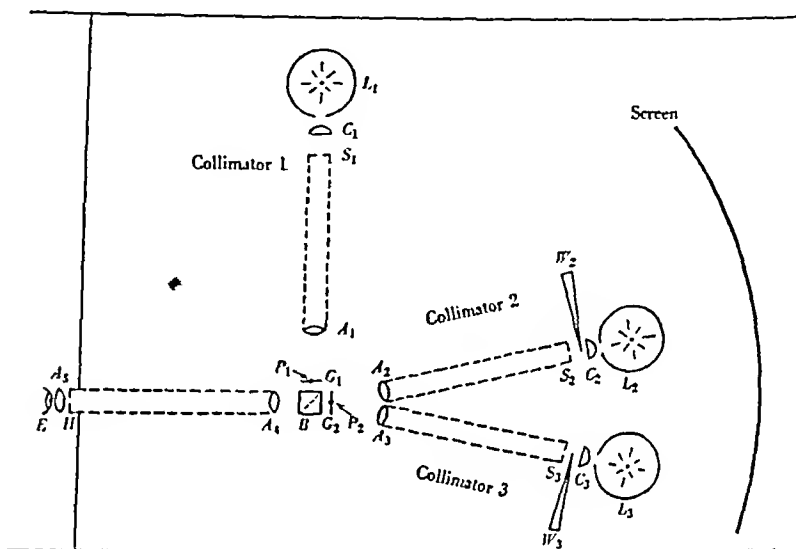


Fig. 1. Diagram of apparatus used.  $L_1$ ,  $L_2$  and  $L_3$ , 48 W. 12 V. motor headlights inside lamp houses with a slit in each to direct light down the respective collimators.  $C_1$ ,  $C_2$  and  $C_3$ , cylindrical lenses to concentrate light through slits  $S_1$ ,  $S_2$  and  $S_3$  on to collimating lenses  $A_1$ ,  $A_2$ , and  $A_3$ .  $G_1$  and  $G_2$ , gratings opposite end and side of Lummer-Brodhun cube  $B$ .  $A_4$ , telescope lens to concentrate light from surface of Lummer-Brodhun cube to give Maxwellian view in eye  $E$  through hole  $H$  at end of telescope.  $A_5$ , lens used to form images of slits  $S_1$ ,  $S_2$  and  $S_3$  when calibrating collimators by lines of known wave-lengths.  $P_1$ , pivot round which collimating system 1 turns.  $P_2$ , pivot round which collimating systems 2 and 3 turn.  $W_2$  and  $W_3$ , photometric wedges for altering intensity of lights in collimating systems 2 and 3. Straight lines surrounding the instrument show the screens which prevent stray light from affecting the observer's eye at  $E$ . Needles above slits  $S_1$ ,  $S_2$  and  $S_3$  were illuminated by light from the lamp houses and their shadows were focused by lenses above lenses  $A_1$ ,  $A_2$  and  $A_3$  on to the calibrating scale behind lamp houses  $L_2$  and  $L_3$ . The shadows from needles on collimating systems 2 and 3 were reflected by a mirror vertically above grating  $G_2$ . The shadow of the needle on collimating system 1 was reflected on to the same scale by a totally reflecting prism vertically above grating  $G_1$ . The calibrating scale is really much farther away (2 m.) but is placed closer on the diagram for convenience.

The wave-length of the light was determined from a linear millimetre scale which had been calibrated in the following way. The shadow of a needle above each slit ( $S_1$ ,  $S_2$  and  $S_3$ ) was formed by a lens mounted above and secured to the corresponding collimator. The images of the needles

above the two collimators opposite to the telescope were reflected by a plane mirror placed vertically above the grating  $G_2$ . The image of the needle above the collimator at right angles to the telescope was reflected by a totally reflecting prism placed above the grating  $G_1$ , opposite to the side of the cube. Thus all three images could be seen on the same screen. The screen was curved to form an arc of a circle of 2 m. radius centred on the cube. The scale was calibrated by observing the position of the image of the needle when the slit of the collimator seen through the eyepiece,  $A_5$ , was visible as a narrow white band, then the collimator was moved round its pivoting point until a known band of the neon vapour or copper arc lamp was visible. Thus, by knowing the movement of the image in millimetres, the wave-length of the light visible through the cube could be calculated. Approximately each millimetre of movement corresponded to a difference of 9 Å.



Fig. 2.



Fig. 3.



Fig. 4.

Fig. 2. Appearance of Lummer-Brodhun cube when illuminated from the side.

Fig. 3. Appearance of Lummer-Brodhun cube when illuminated from the front.

Fig. 4. Appearance of field of view when Lummer-Brodhun cube is cut down by masking top and bottom portions.

The eye aperture ( $H$ ) of the telescope was a hole 0.34 mm. in diameter placed at the focus of the telescope lens,  $A_4$ ; hence the cube was seen by the Maxwellian view. During calibration of the collimators an eyepiece ( $A_5$ ) was placed between the hole in the telescope and the eye so that the slit and the known calibrating bands could be seen.

The slits of the collimators were illuminated by 48 W. motor head-lamp bulbs connected in series and run from the mains through a resistance at a constant current of 4 amp. Cylindrical lenses ( $C_1$ ,  $C_2$  and  $C_3$ ) condensed the light from the filaments on to the slits. The lights and apparatus were adequately screened from the subject, who was in a canopy of plywood painted black with the telescope protruding through a hole in the front of the canopy. The various controls for moving the collimators were rods projecting through the same surface, and disks on the ends of the rods provided an easy means of adjustment.



## EXTENT AND POSITION OF THE "BLUE-GREEN" REGION

For the first portion of the work only one collimator was used. The subject was asked to manipulate the controls until the colour appeared to be blue-green. He was instructed to move the collimator until he considered the colour to be distinctly greenish, then back until the colour appeared distinctly bluish. By noting the scale readings corresponding to these two positions the extent of the "blue-green" region of the spectrum was determined. Five readings of each position were made and the median is taken as the most likely figure. The average of the two medians is recorded as the "mid blue-green", that is, the centre of the "blue-green" region.

PROPORTION OF "BLUE" AND OF "GREEN" TO MATCH  
A GIVEN "BLUE-GREEN"

For the second part of the work the collimator directing light on the side of the cube was set to give light of a fixed wave-length (4934 Å), which was thought to be close to the "mid blue-green" position. The other two collimators were set one on each side of the normal to the front face of the cube to give respectively a blue (4884 Å) and a green (5031 Å) light which were combined in the telescope. Between the lights and the slits of these two collimators were photometric wedges ( $W_2$  and  $W_3$ ) controlled by handles projecting through the plywood screen. The subject altered the intensities of the "blue" and "green" until the whole field appeared a uniform blue-green. The total intensities of the blue and green were measured by a photoelectric cell and the intensities, when the field was matched, were determined from the readings of the galvanometer and the transmission through the photometric wedges. It was intended to compare the relative illuminations for the different mixtures required by different individuals to match the same "blue-green" light. This mixture corresponds to the Rayleigh mixture of "red" and "green" to match a yellow colour.

## RESULTS

*Extent and position of the "blue-green" region*

One hundred cases were examined. The first ten cases were examined, each on ten separate days. On each day the effects of dark and of light adaptation were tried. There was no appreciable difference between the results obtained in the dark and light adapted conditions, possibly because the brightness of the fields soon produced light adaptation from the dark adapted state.

Fig. 5 shows the results for ten separate sittings with ten individuals who were not dark adapted. Each record is the median of five consistent readings. It will be seen that some subjects systematically select a definite region, whilst others systematically select a different band of

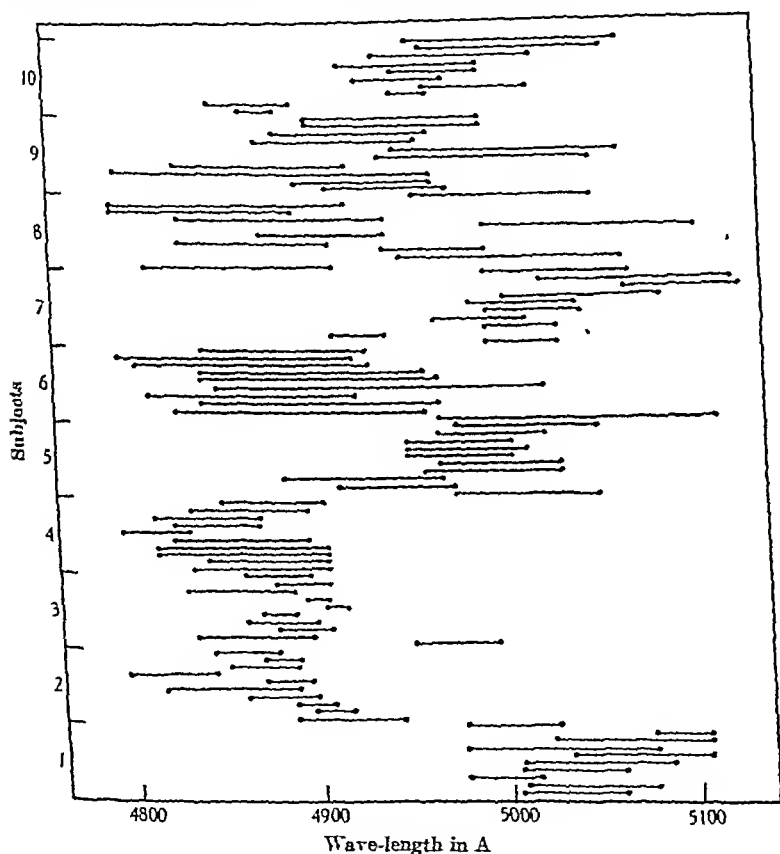


Fig. 5. Limits of "blue-green" region. Records of readings on ten separate days for ten different individuals. Some subjects consistently select one region of the spectrum, others select a different region; still others make different selections on different days. The frequency curve for these 100 readings shows evidence of two maxima.

wave-lengths. Others still show a tendency to choose different bands on different days for their selection of "blue-green". In the choice of "yellow" there is not the same tendency to select different bands. Although those with defective colour vision select a much wider range for yellow, the "normal" region is usually included. The frequency diagram (interrupted line in Fig. 7) for the 100 observations on these ten

cases suggests two maxima, although the readings for the whole 100 cases do not show so great an indication of two maxima. The double maximum for these ten cases may be due to the fact that the ten readings for each case may have "weighted" the results unduly. Nevertheless,

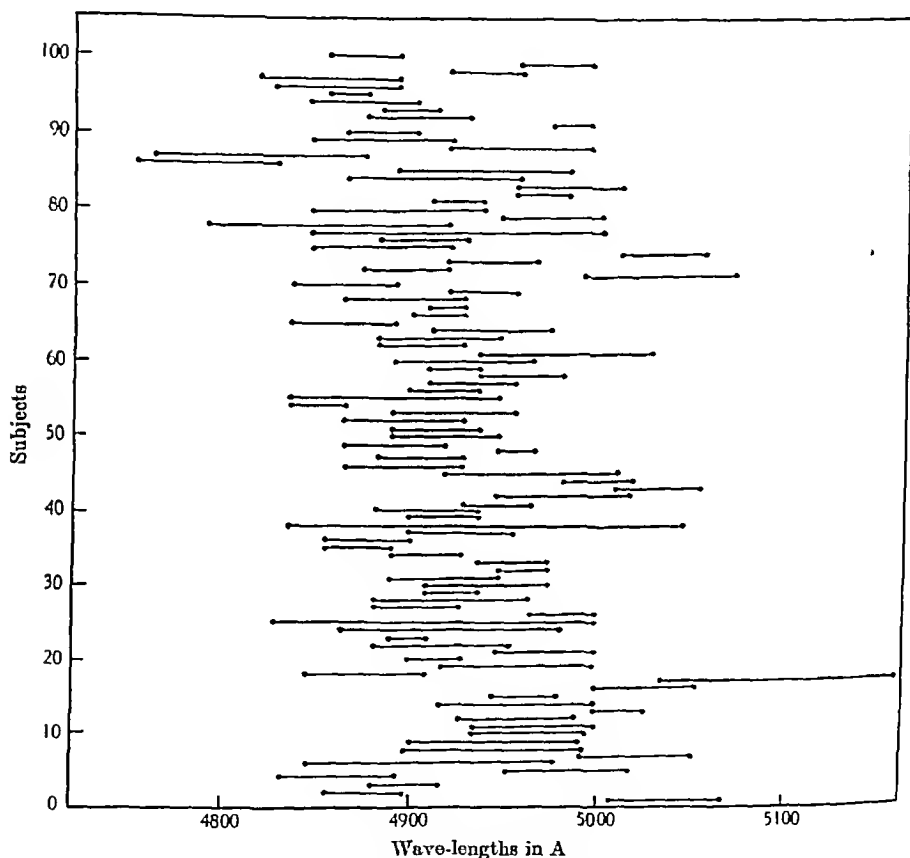


Fig. 6. Limits of "blue-green" region. Records of readings on 100 separate individuals. The first ten records are made from the averages of the ten readings on ten separate days shown in Fig. 5.

the fact that several subjects consistently select a different region for the "blue-green" is probably significant. The fact that some subjects select one region on one day and another on a different day may mean that the selection of different regions is not constitutional but is due to some adventitious factor. We attempted to keep the experimental conditions constant, but it may have been that the selection of different regions by

different individuals may have been due to some constant experimental difference which we failed to observe.

As the results obtained with the first ten cases did not show any fixed difference between the dark and light adapted states, the remaining ninety cases were examined at only one sitting and they were not kept for half an hour in the dark to produce adaptation. Fig. 6 shows the positions and extent of the regions which were marked as lying between a distinctly bluish and a distinctly greenish blue-green. The average

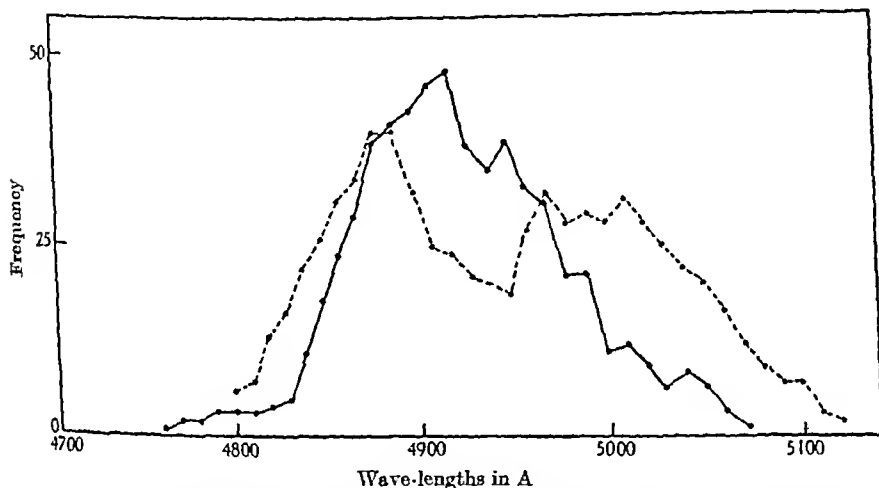


Fig. 7. Frequency diagram showing the number of lines above the various wave-lengths at 10 Å intervals. The interrupted line shows the results for the first ten subjects (Fig. 5). The full line shows the results for the whole 100 subjects (Fig. 6).

width of the band was  $60.47 \pm 33.36$  Å and the middle of the band was at  $4928 \pm 51.3$  Å. For this diagram the averages of the ten separate days for the first ten cases were used.

Fig. 7 is constructed by adding the number of lines above different wave-lengths at 10 Å intervals: this gives a frequency diagram. The diagram suggests that there is much greater spread in the observations than with the "yellow" region, in fact there is a slight indication of two maxima, especially in the curve for the first ten cases.

The ratios of "blue" and "green" to match the fixed "blue-green" were variable, and this part of the work requires reinvestigation by better methods.

## DISCUSSION

The average width of the "blue-green" region is not greater than that of the "yellow" region, but its position is more variable (a standard deviation of 51.3 Å against 38 Å). This result agrees with the fact that the wave-length discrimination curve shows a minimum difference approximated in value to that in the "yellow" region, but the low differential threshold spreads over a wider range of wave-lengths.

Defective colour vision as judged by the Ishihara test does not affect the width or position of the "blue-green" region.

## SUMMARY

An apparatus is described by means of which the "blue-green" region of the spectrum was demarcated by 100 subjects.

The average width of the "blue-green" region of the spectrum is  $60.47 \pm 33.36$  Å and its mid-position is at  $4928 \pm 51.3$  Å. For "normal" persons the corresponding measurements for the "yellow" region are  $67 \pm 29$  and  $5853 \pm 38$  Å respectively.

There is some indication that different individuals may place their "blue-green" regions at different wave-length bands of the spectrum, whilst the "yellow" region seems more uniform in position for normal people.

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## THE WEIGHT OF THE CHROMOPHORE CARRIER IN THE VISUAL PURPLE MOLECULE

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Visual purple, because of its physical and chemical properties, is now generally considered to be a protein. For example it has a high molecular weight and is precipitated by water-soluble organic solvents. It exhibits cataphoretic properties with an isoelectric point [Broda, Goodeve, Lythgoe & Victor, 1939] as well as an ultra-violet absorption curve characteristic of proteins [Krause & Sidwell, 1938; Goodeve, Lythgoe & Wood, unpublished]. It can be salted out of solution with sodium sulphate, apparently unaltered, and the kinetics of its thermal decomposition [Lythgoe & Quilliam, 1939] also indicate its protein nature.

Visual purple appears to belong to the class known as "chromoproteins", that is, coloured proteins. The electron or electrons which absorb light and thus give rise to colour are more or less localized with a group of atoms and this group is called a "chromophore". There may be a number of chromophores in one molecule of a chromoprotein. The chromophore can sometimes be removed from the compound by organic solvents leaving behind the colourless protein. With visual purple non-polar organic solvents, such as light petroleum and carbon tetrachloride, have no effect at all either on the solid form or the solution, while others, such as ether or chloroform, decolorize the visual purple and extract a yellow substance which is probably derived from the chromophore. Wald's [1936] results indicate that the chromophore of visual purple is carotenoid in nature, and that light acts by loosening the bond between it and the protein, so that the former becomes more easily soluble in organic solvents.

Any treatment of visual purple which splits the chromophore off the protein results in the destruction of its colour, so that it seems that the characteristic properties of this substance are found only when the chromophore and protein are associated. It must, therefore, be assumed that both in the retina and in solution, visual purple is always in the form of a conjugated protein.

In a chromoprotein the chromophore is commonly the reactive part, while the bulky protein seems to act mainly as a support—for instance, the oxygen is carried by the chromophore of haemoglobin. If  $M$  is the molecular weight of the chromoprotein, and  $P$  the number of chromophores attached to each molecule of protein, then  $R$ , the molecular weight of that part of the chromoprotein which contains one chromophore, is given by

$$R = M/P,$$

$R$  will be called the "carrier weight" of the chromoprotein. (It would be better to define  $R$  through the molecular weight of the protein itself rather than that of the chromoprotein. At present this is impossible for most chromoproteins, since we do not know the molecular weight of the isolated protein. Fortunately the difference in the values obtained in the calculations made below would not be significant, since the chromophore is so much lighter than the protein.)

*Carrier weights and the Svedberg unit.* The number of chromophores per unit weight of chromoprotein has been determined for several chromoproteins by various workers. The values of the carrier weights,  $R$ , have been calculated from these data, and are given in Table I, together with the chemical nature of the chromophore, upon which the calculations are based. In erythrocytorin, for example, one gram-equivalent of the prosthetic group with one gram-atom of iron is contained in 17,500 g. of chromoprotein.

The value of the fundamental protein unit discovered by Svedberg [see, e.g., 1937; Svedberg & Pedersen, 1940] is about 17,600. It is a striking fact that six of the chromoproteins investigated (Table I) have carrier weights lying between 16,200 and 18,700, and therefore virtually equal to the Svedberg unit. The carrier weight of the remaining chromoproteins exceeds this value but it is never less, suggesting that at least one Svedberg unit is required for the support of one chromophore.

*The carrier weight of visual purple.* In the chromoproteins in Table I the concentration of the active groups in a solution can be determined directly. On the other hand, for visual purple no characteristic chemical reactions are known and the small amounts so far available have not

TABLE I. *Carrier weights.* (The number of grams of protein per gram-equivalent of chromophore)

Chromoprotein	Carrier weight <i>R</i>	Chemical nature of the chromophore (on which <i>R</i> is based)	References
Hæmoglobin (horse)*	16,200	Haeme	Theorell [1932]
Myoglobin*	16,200	An Fe compound	Theorell [1932]
Erythrocytin (cyclostomata)	17,500	An Fe compound	Svedberg & Hedenius [1934]
Cytochrome <i>C</i> *	16,400	An Fe compound	Theorell [1935 <i>a</i> ]
Hæmocuprein*	18,700	A Cu compound	Mann & Keilin [1938 <i>a, b</i> ]
Hepatocuprein	18,700	A Cu compound	Mann & Keilin [1938 <i>b</i> ]
Hæmocyanin ( <i>Helix pomatia</i> )	25,900	A Cu compound	Hernler & Philippi [1933]
Hæmocyanin ( <i>Busycon canaliculatum</i> )*	25,900	A Cu compound	Hernler & Philippi [1933]
Hæmocyanin ( <i>Octopus vulgaris</i> )*	25,400	A Cu compound	Hernler & Philippi [1933]
Hæmocyanin ( <i>Loligo pealei</i> )*	24,300	A Cu compound	Hernler & Philippi [1933]
Hæmocyanin ( <i>Limulus polyphemus</i> )	36,700	A Cu compound	Hernler & Philippi [1933]
Hæmocyanin ( <i>Homarus americanus</i> )	34,000	A Cu compound	Hernler & Philippi [1933]
Polyphenol oxydase	31,700	A Cu compound	Kubowitz [1937, 1938 <i>a, b</i> ] Keilin & Mann [1938]
	21,200		
Chromoproteins not containing metals			
Phycocyan } Phycocerythrin }	~30,000	Phycobilin	Lemberg [1929, 1930]
Yellow ferment*	{ ~70,000 ~80,000	Flavin	Theorell [1935 <i>b</i> ] Kekwick & Pedersen [1936]
Oroverdin	144,000	Astaxanthene	Kuhn & Sorensen [1938]

\* Crystallized preparations.

permitted analyses to be made. The concentration of the chromophore can, however, be determined from the optical density of the solution and the extinction coefficient. This latter has been deduced by Dartnall, Goodeve & Lythgoe [1938] from the photosensitivity which can be measured directly and is the product of the extinction coefficient and the quantum yield (the quantum yield gives the number of chromophores decomposed by each quantum of absorbed light). Thus at  $\lambda=500$  m $\mu$  the extinction coefficient is found to lie close to  $9 \times 10^{-17}$  sq. cm. per molecule. The decadic molar extinction coefficient  $\epsilon$  is obtained from this by including Avogadro's number and converting to decadic logarithms and



concentrations per litre; it has the value  $2.3 \times 10^4$ . The concentration of chromophores in g.mol. per l. is given by  $c = d/\epsilon l$ , where  $d$  is the optical density of the solution and  $l$  the thickness of the layer.

### METHODS

The solutions were prepared by the method described by Lythgoe [1937]. Exp. 5 was made the basis of the calculation which follows, and in this the ratio of the density at  $500 \text{ m}\mu$  to that at  $400 \text{ m}\mu$  was 3.1, indicating a solution of high purity. The optical density of the solutions was measured on a Hilger Spekker photometer.

*The weight of protein. Method 1.* The optical density having been determined, the solutions were dialysed for 2 days at about  $3^\circ \text{C}$ ., and electro dialysed for 12 hr. to remove digitonin, salts and part of the phospholipins present [Krause, 1937; Broda, Goodeve & Lythgoe, unpublished]. During electro dialysis, nearly all the visual purple was precipitated. Subsequently the remainder of the protein was precipitated by the addition of alcohol. The protein was then centrifuged down, washed with water, alcohol and chloroform, dried at  $105^\circ \text{C}$ . and weighed. The N content of the protein was determined by the micro-Kjeldahl method.

*Method 2.* The solution was bleached and the water evaporated in contact with  $\text{CaCO}_3$  at room temperature. The residue was extracted alternately with chloroform and alcohol. The united extracts contained yellow colouring matter and the digitonin. The residue, which contained the whole of the protein, was treated with dilute acetic acid (part of the protein remained undissolved). The solution was boiled with alcohol and left to stand, when the whole of the protein precipitated. It was centrifuged down and washed, dried and weighed as described in method 1. A disadvantage of method 2 is that the residue contains appreciable quantities of Ca salts.

### RESULTS

The results of five determinations of the carrier weight,  $R$ , are given in Table II, together with the values from which they were calculated. The values of  $P$  were obtained by dividing the molecular weight of visual purple, 270,000 [Hecht & Pickels, 1938], by the value of  $R$ .  $R$  varies between 43,000 for the first experiment and 34,500 for the last and best experiment, but these values are subject to certain errors, most of which tend to raise the value above what it should be.

The protein prepared by either method was always slightly yellow, showing that it was not pure: it probably contained some non-protein,

TABLE II. The carrier weight,  $R$ , of visual purple (uncorrected) and the number of chromophores,  $P$ , contained in one molecule of chromoprotein

Exp.	Method	$\tau$ c.c.	$w$ g.	$d$ (500 m $\mu$ ; $l=1$ cm.)	$c$ ( $d/\epsilon l$ ) in g.mol./c.c.	$R$ ( $w/\tau c$ )	$P$
1	2	2.11	$6.4 \times 10^{-4}$	0.16	$0.70 \times 10^{-8}$	43,000	6.2
2	2	2.60	$7.8 \times 10^{-4}$	0.18	$0.78 \times 10^{-8}$	38,000	7.2
3	2	2.85	$84.8 \times 10^{-4}$	1.68	$7.3 \times 10^{-8}$	41,000	6.6
4	1	2.85	$19.7 \times 10^{-4}$	0.40	$1.8 \times 10^{-8}$	39,000	6.9
5	1	2.85	$72.1 \times 10^{-4}$	1.67	$7.3 \times 10^{-8}$	34,500*	7.8*

Exp. 5 is the most accurate since the preparation was a good one and electro dialysis was very thorough.

\* See text for correction based on N content.

such as phospholipins or derivatives of the chromophore, both of which are yellow. Since the free chromophore has a molecular weight which is small compared with that of a protein, its presence or absence will make no appreciable difference, but if lipins in large quantities are still present they would make a significant addition to the final weight. Lipins are often tenaciously held by proteins [see, e.g., Sorensen, 1930].

Some allowance can be made for errors due to impurities such as phospholipins, which are poor in N, by determining the nitrogen content of the residue. Proteins usually contain 15–19% N, but the solid residue in Exp. 5 had a N content of only  $12.0 \pm 0.1\%$ , so that the weight of the protein ( $w$ ) must be less than 80% of that given in the table. If the values of Exp. 5 are corrected on this assumption, the value of  $R$  becomes 27,600 or a little less and the value of  $P$  becomes 9.8 or a little more. Consideration of a N content of the impurities would further reduce the value of  $R$ . For example, if the impurities are assumed to contain 1.8% N (the ordinary N content of phospholipins), the value of  $R$  would be less than 26,500 and of  $P$  more than 10.1.

If any bleaching of the visual purple solutions occurred before the optical density was taken, another error would be introduced. In fact very little bleaching can occur since the solutions do not show the indicator properties associated with the bleaching product indicator yellow. Another error might arise from the presence of proteins other than visual purple, although no such protein has yet been isolated.

Each of these errors acts in the same direction, namely, to raise the apparent value of  $R$ . (In the calculation of the chromophore concentration it was assumed that the quantum efficiency of the bleaching process is one. It is just possible, however, that the value is slightly below unity [Schneider, Goodeve & Lythgoe, 1939], and if this were so, the calculation

concentration would be too high, and the observed value of  $R$  too low.) The carrier weight of visual purple is, therefore, probably somewhat below 26,500, a value which agrees reasonably well with that of the Svedberg unit considering the approximate nature of the calculation. Visual purple may share with haemoglobin, erythrocrucorin, etc. the property of carrying a maximum amount of chromophore, i.e. one per Svedberg unit of its protein.

### *Visual purple in the retina*

Using the results given above one can calculate the amount of visual purple in the outer limbs of each rod, provided the number of rods used in the preparation of a solution is known. In a separate experiment the rod concentration was determined by preparing a suspension of rods in the usual way and placing a small drop of the suspension in a haemocytometer. (Owing to the war it was not possible to use *Rana esculenta* in this as in Exps. 1-5. The rod-count was made on the retinae of *R. temporaria*.) It is not possible to make a straight count as it is with erythrocytes, since the rods were broken into various lengths. They could, however, be graded visually into whole, three-quarter, half and quarter lengths, and the count of each one of these units made. Knowing the total volume of the solution, its rod content was calculated. The suspension contained both inner and outer limbs of rods and, in order to make a correction for the inner limbs, a drop was withdrawn and mixed with a drop of 1% osmic acid solution. After 24 hr. the outer limbs could be clearly distinguished on account of their darker staining. From the results of a differential count, the number of outer limbs in the suspension was calculated. It was found that ten retinae yielded 5,700,000 outer limbs. A solution of visual purple was made from the suspension of rods in the usual way. No visual purple was lost in the various reagents with which the rods were treated, but in order to ensure that the extraction by digitonin was complete, the residues after the first extraction were again treated with digitonin for a long period. The density of this second solution was found to be small, showing that the first extraction was almost complete. The value obtained was added to that of the first extract, giving 1 c.c. of solution having a total optical density of 0.814 for a layer 1 cm. thick, or one-tenth c.c. of solution per retina. If this amount of solution had been spread over an area of 0.15 sq. cm. (the approximate area of a frog's retina), clearly then the density would have been 0.54. This, therefore, was the original density of the visual purple in a dark adapted frog's retina.

By the use of the formula,  $c = d/\epsilon l$ , the yield of visual purple from one retina is found to be  $3.5 \times 10^{-9}$  g.mol., whilst the yield from one rod is  $3.5 \times 10^{-9}/570.000$  or  $6.1 \times 10^{-15}$  g.mol. of the chromophore. (Owing to improved methods of extraction, the yield per retina is higher than that given previously, namely  $10^{-9}$  g.mol. per retina [Lythgoe & Goodeve, 1937].) Avogadro's number is  $6.06 \times 10^{23}$ : there are, therefore,  $6.1 \times 10^{-15} \times 6.06 \times 10^{23}$  or  $3.7 \times 10^9$  chromophores of visual purple in each rod, or  $3.7 \times 10^9/10$  molecules of the whole chromoprotein.

If the carrier weight for visual purple is 26,500, its weight in one rod will be  $6.1 \times 10^{-15} \times 26,500$  or  $1.62 \times 10^{-10}$  g. Since the sp.gr. of visual purple will be much the same as that for other proteins, namely 1.3, the volume occupied by this weight will be  $1.24 \times 10^{-10}$  c.c. The outer limbs of the frog's rod are  $5 \times 10^{-3}$  cm. long and  $6 \times 10^{-4}$  cm. in diameter [Schmidt, 1938]; the volume is, therefore,  $1.4 \times 10^{-9}$  c.c. The proportion of the volume of the outer limb occupied by the visual purple appears therefore to be 8.9%, but, owing to experimental errors and to the approximate nature of the assumed values, all we are justified in saying is that the volume occupied by the visual purple lies between 5 and 10%.

#### SUMMARY

The carrier weight of a chromoprotein is defined as the molecular weight of chromoprotein containing one chromophore. Six of the known chromoproteins have a carrier weight equal to the value of the Svedberg unit, namely about 17,600: in other chromoproteins the value may be greater but it is apparently never less.

The chromophore concentration in a solution of visual purple was deduced from the optical density and the protein weighed. It was found that there are more than 10 chromophores attached to each molecule of protein, and the carrier weight is somewhat less than 26,500, a value not far from the Svedberg unit.

There are over  $10^9$  chromophores in the outer limbs of the rods and the chromoprotein visual purple occupies 5–10% of the volume.

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# THE ACTION OF THE ANTIDIURETIC PRINCIPLE OF POSTERIOR PITUITARY EXTRACTS ON THE URINE EXCRETION OF ANAESTHETIZED ANIMALS

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EXTRACTS of the posterior pituitary gland are known to have two actions on the secretion of urine. Very small doses inhibit the water diuresis of normal mammals. There is substantial evidence that this action is produced by a specific effect on the tubular epithelium. The second—historically earlier—known action of post-pituitary extracts is an increase of the urine flow in anaesthetized animals. This increase is produced by doses which are larger than those used for the production of the anti-diuretic effect, it lasts from 10 to 30 min. and in the experience of the majority of observers is not followed by an inhibition of the urine flow.

The diuretic action of post-pituitary extracts in anaesthetized animals has been explained in various ways. Some authors [Magnus & Schäfer, 1901; Hoskins & Means, 1913; Schäfer & Herring, 1908] thought it due to "a specific stimulation of the renal cells" [Sharpey-Schäfer, 1926]. They arrived at this conclusion because (1) the increased urinary output in some cases outlasts the rise of blood pressure, (2) tachyphylaxia is more pronounced for the blood pressure than for the diuretic effect, (3) the increase in urine flow is sometimes accompanied by an increase in kidney volume and sometimes not.

Other workers [Houghton & Merrill, 1908; Richards & Plant, 1922; Knowlton & Silverman, 1918; Nelson, 1934] postulate a vascular origin of the diuretic effect. They base their opinion on (1) the close parallel between blood pressure and diuresis, (2) the similarity between the action of post-pituitary extracts and the diuretic action of small doses of adrenaline, (3) the absence of an increase of oxygen consumption during



was used to determine the depression of the freezing point of normal rabbit's blood. Fourteen estimations on four animals gave an average of  $\Delta = -0.548 \pm 0.0215^\circ \text{C.}$  for defibrinated venous blood.

## RESULTS

### *The preparation of an antidiuretic non-pressor extract*

It was reported in a previous paper [Heller, 1939] that heating of a commercial posterior pituitary extract for 90 min. at pH 10.0 and  $99^\circ \text{C.}$  resulted in a preparation which contained  $11.5 \pm 0.85\%$  of its initial pressor action, i.e. the ratio of antidiuretic to pressor activity was approximately 100 to 8. For the purpose of this investigation it seemed desirable to obtain an extract which contained even less pressor activity. For this reason the pressor and antidiuretic potencies of extracts were compared which had been heated for 105 and 120 min. respectively. The following curves resulted (Fig. 1). It will be seen that traces of pressor activity are still present after 120 min. of heating. However, this is relevant only to injections of comparatively large amounts of such extracts, the small amounts used in the present investigation had usually no noticeable pressor effect (Fig. 3).

Clark and Lubs buffers were used to maintain the reaction while heating at pH 10.0. This buffer contains potassium in a concentration which, when injected into spinal cats might, to a certain extent, counteract the pressor effect of post-pituitary extracts [Dawes, 1940]. An isotonic potassium free buffer consisting of boric acid and sodium hydroxide was, therefore, used in some experiments but no essential differences of pressor action were found.

Fraser [1937] and Kuschinsky & Bandschuh [1939] published experiments indicating that post-pituitary extracts contain a diuretic substance which seemed to be identical with the oxytocic factor. The objection could, therefore, be raised that the different strength of the pressor and antidiuretic activities after heating at pH 10.0 is only apparent, viz. due to the inactivation of an antagonistic substance which masked the initial concentration of the antidiuretic principle. This possibility would seem to be supported by the observation that the oxytocic factor is undoubtedly inactivated at pH 10.0 [Gaddum, 1930; Heller, 1939]. However, the post-pituitary extract used to obtain a "non-pressor antidiuretic" preparation contained originally only about 5% of oxytocic activity or, in terms of the doses actually employed in the antidiuretic assay, only amounts of the order of 0.1 of a mU. (oxytocic) per 100 g. rat. Using the



technique of Kuschinsky & Bundschuh [1939] twenty-one groups were injected with 0.15 mU. (oxytocic) and 0.30 mU. (oxytocic), rat but no diuretic effects were observed. It seems, therefore, just

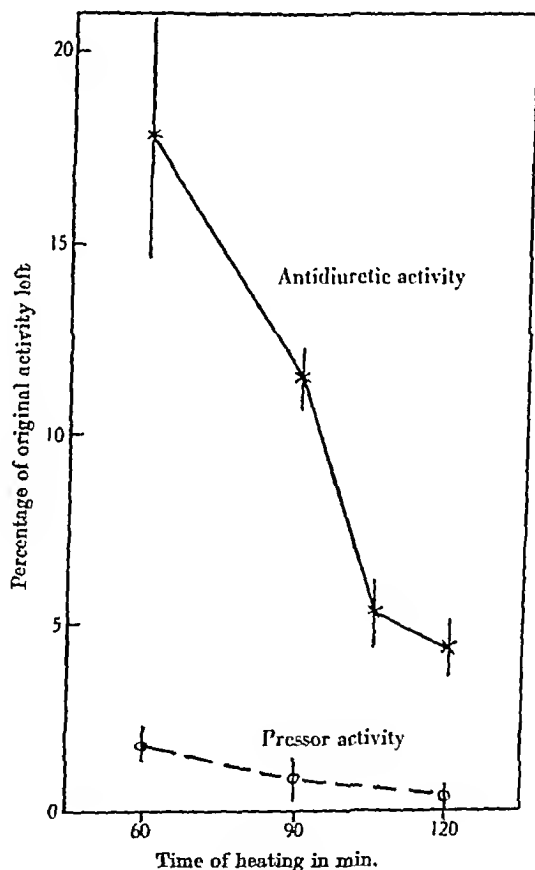


Fig. 1. The inactivation of the antidiuretic and the pressor activity of post-pituitary extracts (pitressin) at 99° C. and pH 10.0. X—X, antidiuretic activity; O—O, pressor activity. The vertical lines indicate the standard error.

to assume that the antidiuretic activity of our heated extracts is the stated percentage of the initial antidiuretic hormone concentration and is not due to an apparent increase of antidiuretic activity caused by the inactivation of a diuretic factor.



cessation of urinary secretion. This (primary) inhibition of urine flow is known to occur—not with regularity however—in anaesthetized animals and has been attributed to a brief ureter spasm immediately after the

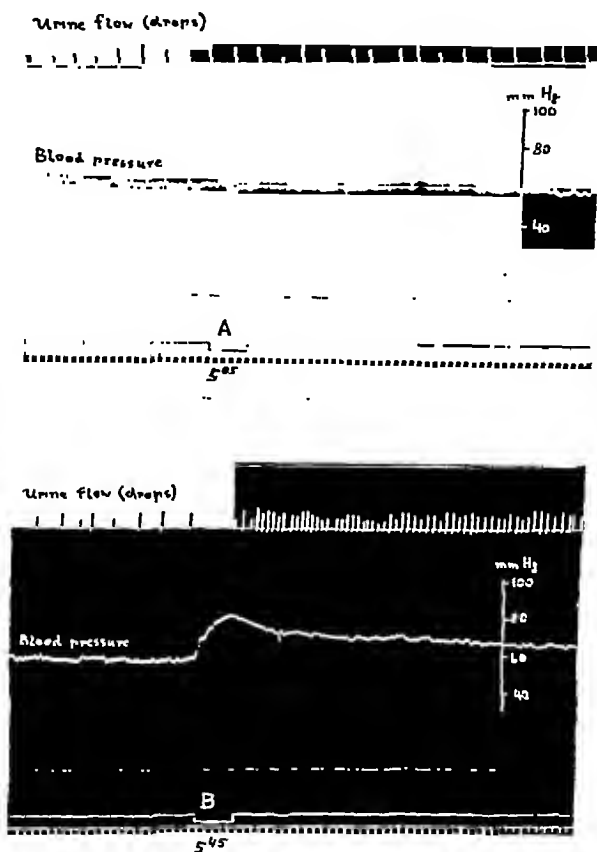


Fig. 3. Comparison of the effect on B.P. and urine flow of an anaesthetized (urethane) rabbit of an intravenous injection of a post-pituitary extract containing pressor and antidiuretic activities in equal proportion with the effect of a non-pressor preparation of the same antidiuretic activity. *Same rabbit as in Fig. 2.* A = intravenous injection of 2000 mU. pitressin heated for 90 min. at 99° C. and pH 10.0. B = intravenous injection of 200 mU. pitressin not heated. Time marker = 5 sec.

intravenous injection [Molitor & Pick, 1924; Mackersie, 1925]. In the present series it was observed in the majority of experiments with pressor post-pituitary extracts. Neither this immediate cessation nor the

subsequent rise in urine flow followed upon an injection of a non-pressor preparation (Fig. 3a) which had been administered 40 min. previously. The non-pressor extract was given first and before the rabbit had received any other post-pituitary extract on that day. This excludes the possibility that the absence of a rise of blood pressure was due to the refractoriness (tachyphylaxia) of the animal after a previous injection of post-pituitary extract. The lack of a diuretic effect concurrent with the absence of a rise of blood pressure is obvious. The short primary inhibition is equally absent. Nor was it found to occur in other experiments with non-pressor extracts.

The experiment recorded in Fig. 3 was performed on an animal which had received 147 c.c. of water by stomach tube before urethane anaesthesia started. Sufficient time (2 hr.) had been allowed for the intestinal absorption of the bulk of this amount [Heller & Smirk, 1932a]. The urinary excretion rate in this and other experiments of the same series was nevertheless low. The same results regarding the action of pressor and non-pressor antidiuretic preparations on blood pressure and urine flow were obtained when a diuresis was produced by intravenous infusion of hypertonic (10%) solutions of sucrose or sodium sulphate during the anaesthesia.

Neither in these experiments nor where water had been given by stomach tube did a (secondary) inhibition follow the diuretic response. The significance of this finding will be discussed in the following section.

It is clear from these results that the primary inhibition and the increase in the rate of urine flow following upon an intravenous injection of an untreated post-pituitary extract is due to its pressor activity, and not to the antidiuretic principle proper.

#### *The relation of the urinary osmotic pressure to the action of the antidiuretic hormone*

It is well known that the post-pituitary antidiuretic factor ceases to have an inhibitory action on the urine flow of unanaesthetized animals if a certain height of urinary osmotic pressure is reached. The evidence for this rests on two groups of experiments: (1) The antidiuretic hormone does not inhibit a diuresis produced by concentrated solutions of a variety of organic and inorganic substances. The high osmotic pressure of the urine excreted under these conditions has not actually been demonstrated, but it can be deduced from the analytical data given by various authors. (2) Injection of post-pituitary extracts does not decrease the urine flow of "unhydrated" animals. The urinary (morning)

osmotic pressure (expressed as freezing point depressions) of a series of ten unhydrated rabbits on a standard diet of oats, bran and cabbage was estimated, and the following figures found:  $\Delta = -3.556, -2.958, -2.155, -2.170, -2.380, -1.735, -2.205, -2.380, -3.065, -2.825$ , average  $\Delta = -2.543 \pm 0.556^\circ \text{C.}$ , that is to say the urine was in every case strongly hypertonic.

It has been mentioned that injections of a non-pressor but anti-diuretic preparation of post-pituitary extracts failed to reduce the urine flow of deeply anaesthetized rabbits which had received large doses of water prior to the injection of the anaesthetic or which during anaesthesia received intravenous infusions of concentrated (10%) solutions of sodium sulphate or sucrose. It can be shown that under these experimental conditions also the antidiuretic principle fails to act because the urinary osmotic pressures are too high. This has been implicitly assumed by numerous authors [Fee, 1929; Melville, 1936; Smith, 1937; and others]. The present investigation offered the opportunity of a quantitative enquiry into the relation between the antidiuretic action of posterior pituitary extracts and the osmotic pressure of the urine. It seemed of interest to determine the "critical urinary osmotic pressure", i.e. that at which the antidiuretic principle ceases to act.

The effect of anaesthesia and operative measures on the flow and osmotic pressure of the urine is shown in Fig. 4, excretion rates per 10 min. falling from 3.8 to 0.28 c.c. and freezing-point depressions falling from  $\Delta = -0.345^\circ \text{C.}$  to  $-2.910^\circ \text{C.}$  The urine flow increases somewhat half an hour after the operation but still remains on a low level, in spite of the "extra water" (=water load)<sup>1</sup> present in the body. 200 mU. pitressin injected at this stage had no antidiuretic effect. Infusion of a hypertonic solution of sodium sulphate or sucrose considerably increases the excretion rate under deep anaesthesia but has little effect on the osmotic pressure of the urine. That is to say the urine remains hypertonic and the antidiuretic hormone does not act. This result is in agreement with Melville's [1936] finding that the rate of urine flow does not affect the action of the antidiuretic hormone.

An unsuccessful attempt was made to produce a "hypotonic diuresis" in four experiments on rabbits under deep urethane anaesthesia. Fig. 5

<sup>1</sup> The water load in this and the following experiments was calculated as the difference between the volume of fluid administered and the volume of urine excreted. Extrarenal losses, about 10 c.c./hr. [see Heller & Smirk, 1932*b*], do not affect the results appreciably and are not accounted for. In this and the following experiments of this series the blood pressure was recorded during anaesthesia to ensure that a decrease of urine flow was not due to a fall in blood pressure.

represents the one of those experiments, in which the lowest urinary osmotic pressures were observed. The animal received 50 c.c. of tepid water per kg. body weight by stomach tube 3 hr., and again 1 hr. before

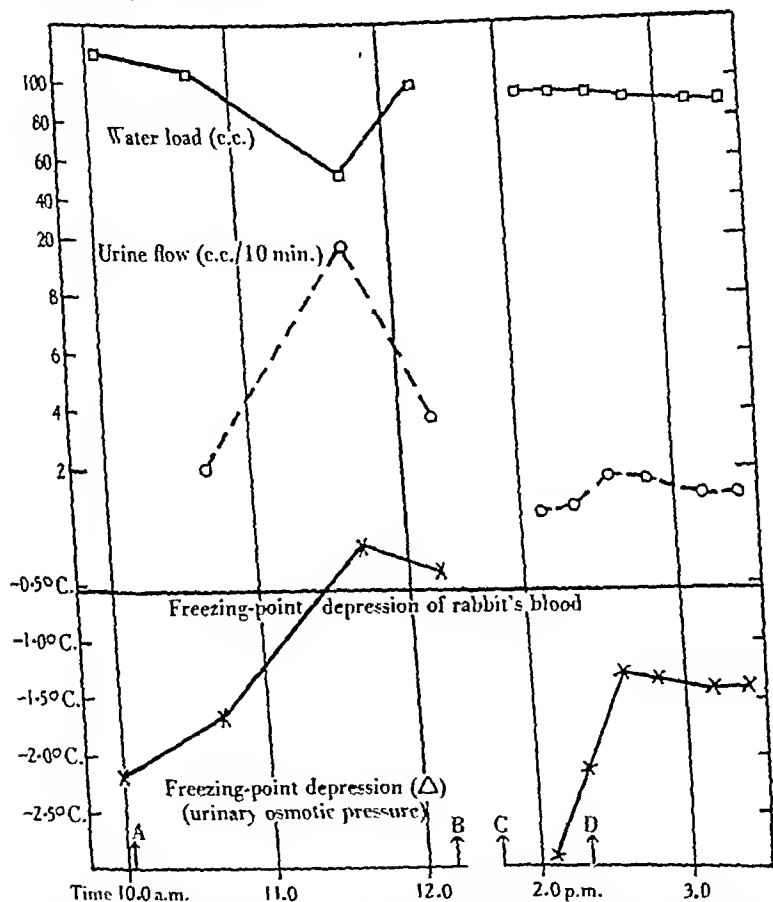


Fig. 4. The effect of anaesthesia and operative procedures on urinary osmotic pressure and urine flow (animal given water prior to anaesthesia. Rabbit ♀, 2900 g. At A 3% of body weight of water by stomach tube. Note that urine becomes hypotonic. At B 2% of body weight of water by stomach tube and 1.5 g. urethane/kg. intraperitoneally. C=operative procedures finished. Note decrease of urinary excretion rate and increase of urinary osmotic pressure. D=intravenous injection of 200 mU. pitressin. Note absence of antidiuretic effect. Urine remains hypertonic. The water load decreases little during anaesthesia, thus denoting the inhibition of diuresis.

anaesthesia started. The osmotic pressure of the urine fell to a minimum value of  $\Delta \approx -0.085$  after this large quantity of water. Anaesthesia and operative measures increased the osmotic pressure to  $\Delta \approx -0.790$ . This

level was more or less maintained even during a rapid intravenous infusion (6 c.c./min.) of a hypotonic solution ( $\Delta = -0.280$ ) of sucrose. The urine flow under these conditions was quite as high as that reached

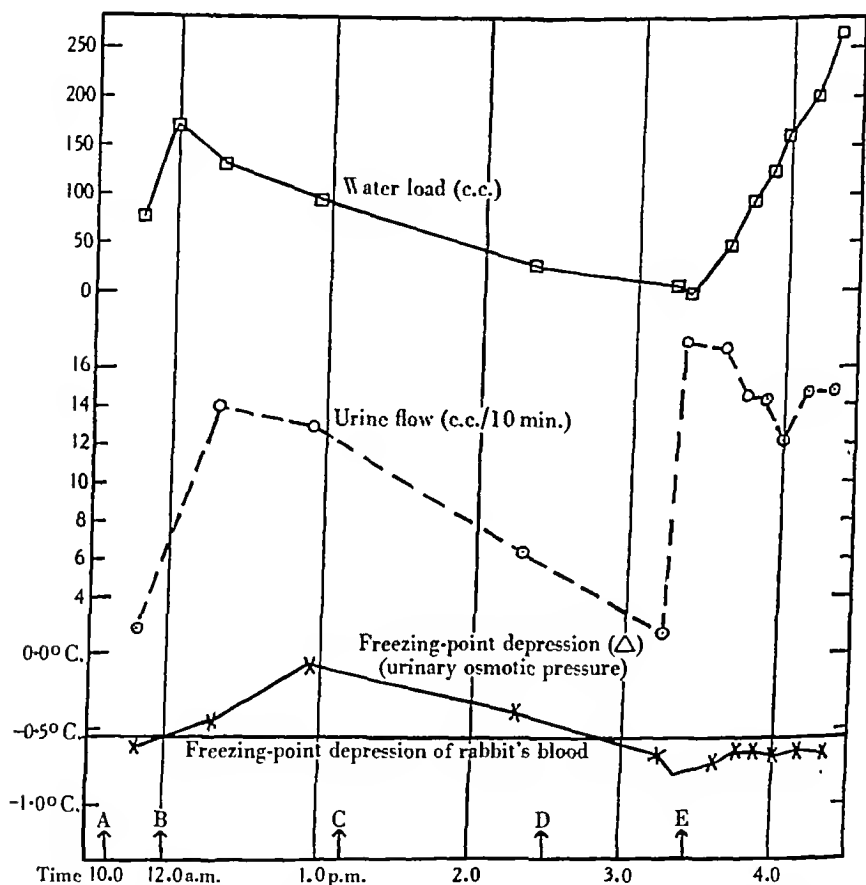


Fig. 5. The effect of anaesthesia and operative procedures on the urinary osmotic pressure and the urine flow of an animal receiving a rapid infusion of a hypotonic solution of sucrose. Rabbit ♂, 2150 g. At A and B 5% of body weight of water by stomach tube. Note low osmotic pressure of urine. At C 1.5 g. urethane/kg. intraperitoneally. D=operation finished. E=intravenous infusion of 4% sucrose started (6.0 c.c./min.). Note hypertonic urine in spite of excretion rate which surpasses that at height of water diuresis.

during the water diuresis of the unanaesthetized rabbit. In spite of the high water load, however, the urine of the anaesthetized animal was not hypotonic.

These results show that once the inhibitory action of the anaesthetic is elicited it can not, or can only to a small degree, be counteracted by blood dilution with a hypertonic or even hypotonic fluid. They suggest that the mechanism underlying the inhibition of water diuresis in anaesthesia is at least in part a renal one.

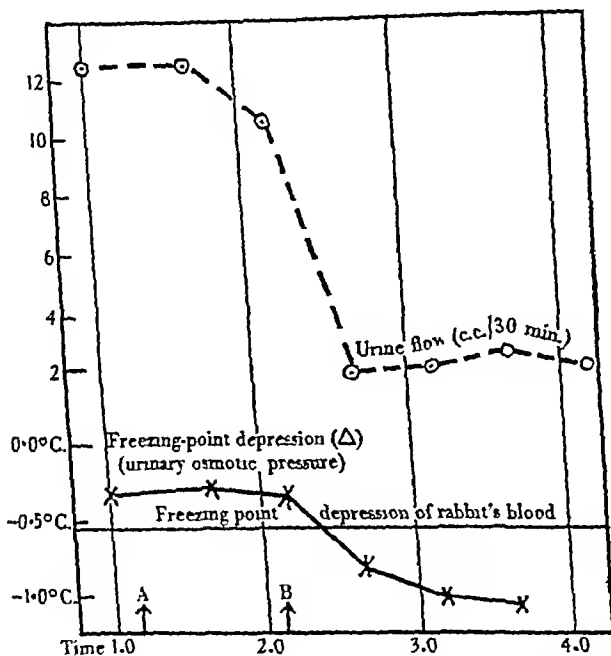


Fig 6. The effect of an injection of pitressin on the urine flow and the urinary osmotic pressure of a rabbit under the influence of a dose of urethane insufficient to inhibit a water diuresis. Rabbit ♀, 1750 g. Not operated (urine obtained by expression of bladder) 5% of body weight of water by stomach tube at 10.0 a.m. and again at 12.0. A = subcutaneous injection of 1.5 g. urethane/kg. Note hypotonic urine after this dose of urethane. See text for description of depth of anaesthesia. B = intravenous injection of 100 mU. pitressin. Note lasting inhibition of water diuresis.

Intraperitoneal injections of the amount of urethane used in the foregoing experiments (1.5 g./kg.) inhibit the water diuresis of rabbits to the same extent if the animals are not operated upon after the anaesthetic has been given. The average urine volume of four such animals in the 2 hr. preceding the injection of the anaesthetic amounted to  $56.1 \pm 22.2$  c.c. as compared with  $5.6 \pm 2.8$  c.c. in the 2 hr. following it. The water load at the beginning of anaesthesia averaged  $125 \pm 26.9$  c.c. The inhibition of the water diuresis after subcutaneous application of urethane (2.5 g./kg.)



was somewhat less pronounced in the small number of animals tested. The figures for three rabbits were:  $34.3 \pm 7.3$  c.c. in the 2 hr. before injection,  $14.1 \pm 1.4$  c.c. in the 2 hr. after injection. Water load:  $148 \pm 24.3$  c.c.

Smaller subcutaneous doses of urethane (1.4 to 1.7 g./kg.) failed to inhibit the water diuresis of unoperated rabbits to any marked extent (Fig. 6), the urine was consequently hypotonic, the antidiuretic principle retained its inhibitory action. Such "smaller doses" of urethane had nevertheless a pronounced anaesthetic effect. For example, the rabbit which yielded the data presented in Fig. 7 showed, at the time of the injection of the post-pituitary extract: a loss of position reflexes, a sluggish corneal reflex, no resentment when its ear was pricked for the intravenous injection of pitressin, and a respiration rate of 34 per min.

The number of experiments performed in these series is not sufficiently great to permit the definition of the "critical urinary osmotic pressure" (i.e. that at which the antidiuretic factor ceases to act) with accuracy. It can, however, be stated that post-pituitary extracts retained a pronounced antidiuretic action at urinary osmotic pressures below  $\Delta = -0.360$  and failed to decrease the urine flow at osmotic pressure values above  $\Delta = -0.800$ .

### DISCUSSION

The experimental evidence presented in this paper shows that non-pressor but antidiuretic preparations of post-pituitary extracts exert no diuretic action in the anaesthetized animals. In fact, such preparations produce no significant change of the urine flow of rabbits under deep urethane anaesthesia in spite of previous ample hydration or infusion of sucrose or sodium sulphate solutions during the experiment. Two main conclusions can be drawn from these results:

(1) The diuretic action in anaesthetized animals of post-pituitary extracts containing both the antidiuretic and the vasopressor activity is due to the latter. The post-pituitary pressor principle can, therefore, be grouped with other pressor substances like adrenaline [Richards & Plant, 1922; Toth, 1937] or veritol [Springorum, 1938] which under similar experimental conditions show a similar diuretic action.

These findings do not necessarily mean that the pressor principle causes the diuretic effect which has been repeatedly observed in badly hydrated, unanaesthetized animals. This diuretic action is usually explained by the increase of the urinary salt excretion caused by post-pituitary extracts [Stehle, 1927], i.e. as an osmotic diuresis. The fact that the antidiuretic principle proper augments the chloride excretion

in the unanaesthetized organism [Heller, 1939] is consistent with this view. However, the results of the present investigation suggest the possibility of an alternative explanation, viz. the diuretic action of the pressor principle under experimental conditions which prevent the action of the antidiuretic factor.

(2) The action of the antidiuretic principle proper is not "reversed", i.e. changed into a diuretic one under the influence of anaesthesia. It is merely abolished if the doses of anaesthetic given are large enough to influence urinary excretion to an extent which raises the osmotic pressure of the urine to values equivalent to freezing-point depressions below  $\Delta = -0.800^{\circ}\text{C}$ .

The antidiuretic hormone may in certain circumstances fail to act even in unanaesthetized animals, for example in badly hydrated animals, and in animals in which diuresis had been produced by concentrated solutions of salts or certain organic substances like urea or sucrose. In both these instances the urine excreted is hypertonic. It can be said, therefore, that in all circumstances whether the animal is anaesthetized or not, the antidiuretic principle ceases to act at high urinary osmotic pressures. This conclusion agrees with the present concept of the physiological role of the antidiuretic hormone in the water metabolism of the mammal. This concept, formed on experimental and morphological evidence [Starling & Verney, 1925; Burgess, Harvey & Marshall, 1933; Smith, 1937] regards the antidiuretic hormone as the means by which a hypertonic urine is produced by the mammalian and probably avian kidney, that is to say, the hormone acts as an agent which in these groups of vertebrates renders the ratio of water to solids excreted more economical. The fact that the antidiuretic hormone ceases to act at a certain osmotic pressure of the urine can, therefore, be regarded as a self-regulating mechanism which prevents the urine to become too concentrated under the influence of the hormone.

#### SUMMARY

1. A non-pressor but antidiuretic extract of the posterior pituitary lobe has been prepared (Fig. 1) and its action on the excretion of urine has been tested.
2. Such preparations have no diuretic action on the anaesthetized animal (Figs. 2, 3). The diuretic effect of post-pituitary extracts containing both the antidiuretic and the vasopressor principle is, therefore, due to the pressor activity.

3. The action of the antidiuretic principle on the urine flow of anaesthetized rabbits depends on the osmotic pressure of the urine elaborated and this in turn on the dose of anaesthetic (urethane) used (Figs. 4, 6). It was found that post-pituitary extracts retained a pronounced antidiuretic action above an osmotic pressure equivalent to  $\Delta = -0.360$  and failed to decrease the urine flow at freezing-point values below  $\Delta = -0.800$ .

I wish to express my thanks to Prof. J. H. Burn for providing facilities in his department. I am indebted to Messrs Parkc, Davis and Co. for a generous supply of posterior pituitary extracts.

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## THE PITUITARY AND THE INSULIN CONTENT OF PANCREAS

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BEST, HAIST & RIDOUT [1939] have shown that the insulin content of the pancreas of rats is reduced by fasting or by feeding fat. In view of the fact that certain pituitary extracts produce a definite change in the insulin content of this gland [Best, Campbell & Haist, 1939] it was of interest to determine the effect on the insulin content of (1) removal of the pituitary, and (2) fat feeding or fasting in hypophysectomized animals.

### METHODS

The methods used were essentially those described by Best, Haist & Ridout [1939]. Female rats were used. Animals weighing between 100 and 200 g. were hypophysectomized, using the retropharyngeal approach. Some of the operations were performed by Dr Jane Russell and some by the author. After operation these rats were kept in individual cages and given as much of a balanced ration and of a 10% sucrose solution as they desired. The daily caloric intake in the control animals was kept as nearly as possible the same as that of the hypophysectomized rats. All of the hypophysectomized animals and some of the "paired-fed" controls lost weight, while some of the latter group made a slight gain.

### RESULTS

The results of the first experiment are shown in Table I. The average normal value for rats fed *ad libitum* was 26.5 units of insulin per group of ten rats. This was the average value obtained on eleven groups of normal rats in a previous experiment. It is evident from these results that while the hypophysectomized rats have a lower average insulin



These indicate that in hypophysectomized animals feeding fat causes a fall in the insulin content of pancreas to a level even lower than that of the "paired-fed" controls. The difference in the appearance of the adrenal glands in the two groups is shown in Fig. 1. This is a photograph of the adrenals from one hypophysectomized and one control group. This finding, along with the failure to gain weight and the absence of any visible pituitary tissue, on careful inspection at autopsy, supports the conclusion that the removal of the pituitary was complete.

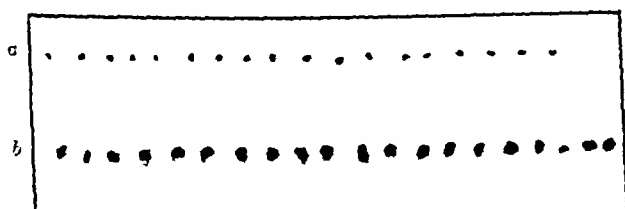


Fig. 1. Photograph of fresh adrenal glands. *a*, adrenals from hypophysectomized rats fed fat for 7 days. *b*, adrenals from control "paired-fed" rats fed fat for 7 days.

In the report of Best, Haist & Ridout [1939] it was shown that feeding a balanced diet to rats starved for 1 week led to a restoration of the insulin content of pancreas to a normal value. Table III shows

TABLE III. Animals fed fat for 7 days, then balanced diet for 7 days

	Hypophysectomized rats	Paired-fed controls
Days after operation	32	—
No. of rats	8	8
Weight (g.).		
Before operation	1193	1229
Before fat feeding	1143	1303
After fat feeding	991	1171
Final	1018	1168
Insulin content of pancreas:		
Unit- group of 10 rats	29.5	26.8
Units 1000 g. initial weight of rats	19	18

the effect of feeding a balanced diet to hypophysectomized rats that had previously been fed fat only, for 1 week. The hypophysectomized animals were first fed fat for 7 days as in the previous experiment and then were given a balanced ration plus 10% sugar solution *ad libitum* for 7 days. The control group received as nearly as possible the same diet and the same caloric intake as the hypophysectomized group before and during the test. At the end of 7 days on the balanced diet the pancreases were removed. It is evident that feeding a balanced diet

restores the insulin content of pancreas. Actually, the final value similar to that of the normal intact animal and is slightly higher than the level in the hypophysectomized group prior to the fat feeding.

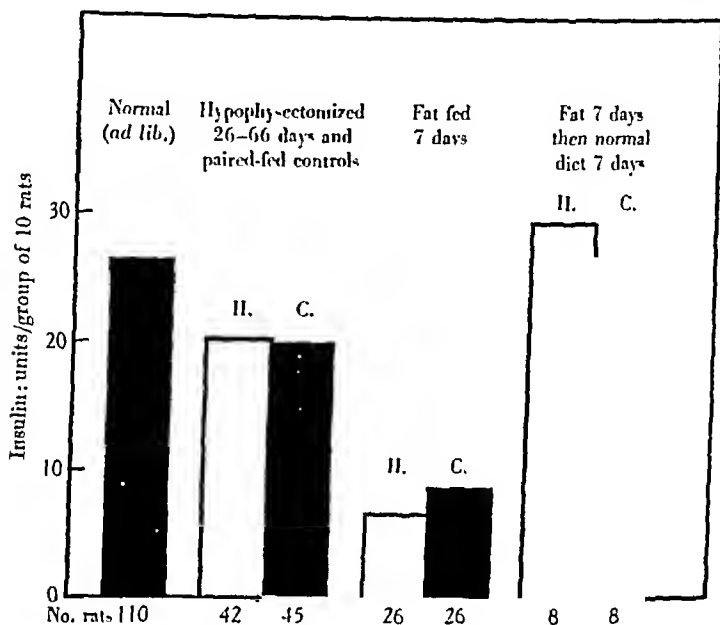


Fig. 2. Hypophysectomized rats. Average results.

### DISCUSSION

The results we have obtained in hypophysectomized animals are well illustrated in Fig. 2. From this it is apparent that hypophysectomy leads to a slight fall in the insulin content of pancreas when the values are compared with those of controls fed *ad libitum*. The values obtained, however, are similar to those in the control animals which received the same caloric intake. It shows also that the fall in insulin content which occurs when fat is fed can be obtained in hypophysectomized animals, and that the feeding of a balanced diet after such a period of fat feeding will restore the insulin content of the pancreas of these animals to normal values.

Chambers, Sweet & Chandler [1935] found that the insulin content of the pancreas of hypophysectomized dogs did not differ from that of normal animals. We have obtained similar results in the rat. They studied the effect of fasting in hypophysectomized dogs and found that after 24-30 days' starvation the ingestion of 25 g. of glucose gave no

rise in respiratory quotient and a definite hyperglycaemia [Chambers, 1938]. These facts indicate that, in some respects at least, the hypophysectomized animals react to starvation in a manner similar to normal ones. The results of our experiments on the insulin content of the pancreas in hypophysectomized rats add weight to the evidence that the pituitary gland is not essential for the effect of fat feeding or of undernutrition on carbohydrate metabolism. It would appear moreover that, *within a wide range, the pancreas can regulate the production and liberation of insulin according to the need for it, in the complete absence of the pituitary gland.*

#### SUMMARY

1. The insulin content of the pancreas in hypophysectomized rats is slightly less than in normal animals fed *ad libitum* but is of the same order as that of controls receiving a similar caloric intake.
2. A fall in insulin content is obtained in hypophysectomized rats when fat is fed.
3. When a balanced ration is given to hypophysectomized rats, which have previously received a diet very rich in fat for 1 week, the insulin content of the pancreas returns to normal within 7 days.

I wish to express my sincere thanks to Prof. C. H. Best for his help and criticism throughout this work. The expert assistance of Miss Helen Bell is also gratefully acknowledged.

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# THE ACTION OF SOME AMINES RELATED TO ADRENALINE: PHENYLALLYLAMINE, PHENYLBUTENYLAMINE, DIPHENYLETHYLAMINE

BY J. A. GUNN AND M. R. GURD

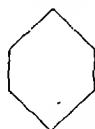
*From the Nuffield Institute for Medical Research, Oxford*

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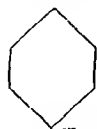
ADRENALINE, 3:4(OH)<sub>2</sub>C<sub>6</sub>H<sub>3</sub>.CH(OH).CH<sub>2</sub>NH(CH<sub>3</sub>), can be regarded as a derivative of the simpler compound  $\beta$ -phenylethylamine, C<sub>6</sub>H<sub>5</sub>.CH<sub>2</sub>.CH<sub>2</sub>.NH<sub>2</sub>. Many of the large number of possible compounds, intermediate between phenylethylamine and adrenaline, have been synthesized and investigated. Also many compounds with simple substitutions, e.g. of methyl and methoxyl groups on the nucleus or side chain, have been examined. A brief review of some of the more important of these compounds and of the relations which have been found to exist between chemical constitution and pharmacological action has recently been given by one of us [Gunn, 1939].

In a series of papers in this Journal an examination has been made of the effect of the following substitutions: (1) methoxyl or methylenedioxy groups in the 3:4-position of the nucleus [Epstein, Gunn & Virden, 1932], (2) a methoxyl group in the  $\beta$ -position in the ethylamine side-chain [Elphick & Gunn, 1934], (3) the combination of phenolic methoxyl groups with an isopropylamine side-chain [Gunn, Gurd & Sachs, 1939], and (4) the reduction of the phenyl to a cyclohexyl nucleus [Gunn & Gurd, 1940].

The present paper gives an account of the actions of the following three compounds, which possess considerable theoretical interest, but none of which has to our knowledge been previously investigated.



(1) Phenylallylamine



(2) Phenylbutenylamine



(3)  $\alpha\beta$ -diphenylethylamine

The object of the first compound was to determine the effect of a double bond in the side-chain, as the particular compound, phenylallylamine, could be compared with phenylethylamine. With regard to the second compound, it is now known that the presence of an  $\alpha$ -methyl group in compounds of the phenylethylamine series has a very considerable, and apparently characteristic, effect on actions of the resulting compounds as is illustrated, for example, by the differences in action of phenylisopropylamine (benzedrine) and phenylethylamine. It was, therefore, of interest to determine whether any similar change of activity occurred when an  $\alpha$ -methyl group was substituted in phenylallylamine. The object of the third compound was to determine what would be the effect of substituting an  $\alpha$ -phenyl group in phenylethylamine, the resulting compound differing from benzedrine only in replacement of a methyl by a phenyl group in the same position. The substances were used in the form of hydrochlorides and the doses given are of the salts.

## RESULTS

### *Toxicity*

The L.D. 50 was determined in mice by intraperitoneal injection. After the approximate dose had been ascertained by administration of graded doses to single animals, the L.D. 50 was determined by the use of not fewer than two groups each of ten animals. The L.D. 50 in g./kg. for the three compounds and also, for comparison, that of  $\beta$ -phenylethylamine and phenylisopropylamine (benzedrine) are given below.

Phenylallylamine	0.23	Phenylethylamine	0.23
Phenylbutenylamine	0.13	Benzedrine	0.12
Diphenylethylamine	0.175		

The toxicity of phenylallylamine is, therefore, almost the same as that of  $\beta$ -phenylethylamine and the addition of an  $\alpha$ -methyl group in the case of both these compounds approximately doubles the toxicity. Diphenylethylamine is slightly less toxic than benzedrine, so that the addition to  $\beta$ -phenylethylamine of a phenyl group in the  $\alpha$ -position increases the toxicity to a less degree than does the addition of a methyl group in this position.

The chief symptoms produced in mice by phenylallylamine were increased motor activity, accelerated respiration and, with lethal doses, mild clonic convulsions. The symptoms were very like those produced by phenylethylamine. A greater degree of central stimulation was produced by phenylbutenylamine, while diphenylethylamine produced

ferent symptoms, motor stimulation being much less marked and, though the animals made more frequent spontaneous movements than normal animals, the movements were ataxic and sluggish.

As the L.D. 50 for mice by intraperitoneal injection is practically the same for benzedrine and phenylbutenylamine, experiments were made with mice with sublethal doses given both by subcutaneous and intraperitoneal injection, in which equal doses of the two substances were given simultaneously to small groups of mice so that the symptoms produced could be compared. The symptoms produced by the two compounds are different. Under benzedrine, the animals displayed incessant restlessness and voluntary movement. Under phenylbutenylamine the animals moved infrequently and then rather stiffly, often with the tail erect. When held up by the tail, the mouse made violent clonic convulsive movements and, when thereafter placed on the floor, often assumed a aleptic condition. Neither with benzedrine nor phenylbutenylamine were there any symptoms of increased spinal excitability.

As for other reasons as well as from its relation to benzedrine, phenylbutenylamine proved the most interesting compound, further experiments in other animals were done to compare its effects with those of benzedrine. Unfortunately the supply of the compound was not sufficient to permit many experiments on the heavier species of animals but the following are examples of a few experiments which could be done.

*Rabbits.* In one experiment two young rabbits, A and B, of the same age were used. Rabbit A received 0.1 g./kg. of benzedrine subcutaneously and rabbit B received the same dose of phenylbutenylamine at the same time, so that the symptoms could be compared. The animal which received benzedrine showed a much greater degree of increased voluntary movement than the one which had received phenylbutenylamine. The former showed the characteristic effects of benzedrine, e.g. frequent stamping of the hindlegs on the floor [Gunn & Gurd, 1940] and incessant rolling at the floor of the cage which went on for over an hour. Rabbit B showed neither of these symptoms. Both animals recovered completely. To see the difference in symptoms might be due to individual differences in response, on a subsequent day, the administrations were reversed, rabbit A receiving the previous dose of phenylbutenylamine and rabbit B that of benzedrine. B now showed the characteristic effects of benzedrine and A did not. The difference in symptoms was therefore not due to individual differences. It might still have been possible that the rabbit was less sensitive to phenylbutenylamine than to benzedrine and that the latter compound would produce stimulant effects similar to those

produced by benzedrine if given in larger doses. That this was not the case was shown by the fact that these effects did not appear even if the dose of phenylbutenylamine were doubled.

Some parallel experiments were made on guinea-pigs with benzedrine and phenylbutenylamine, hypodermically injected. With benzedrine, guinea-pigs rush about less than rabbits or mice. They tend to remain more stationary with coarse tremor of the head and neck. One symptom, which also occurs almost invariably in rabbits, is incessant nibbling of the floor or sides of the tray which may go on for an hour and can be heard over the room. With phenylbutenylamine the latter symptom does not occur. With the latter compound the animals were apprehensive and easily startled, and made occasional short jerky changes of position with marked intention tremor. If the dose were sufficiently great, the animals later fell on the side and remained there, executing without cessation vigorous running or galloping movements which might last for over an hour.

Comparative experiments with benzedrine and phenylbutenylamine on mice, rabbits and guinea-pigs are in sufficient agreement to permit the conclusion that the actions of these two compounds on the central nervous system are not identical; while both stimulate the central nervous system in regions above the spinal cord, the effects of benzedrine suggest rather an action mainly on the motor cortex, while those of phenylbutenylamine are characteristic of an action lower down in the central nervous system.

### *Respiration*

Preliminary experiments on the action of these compounds were made in rabbits anaesthetized with urethane (2 g./kg.), subcutaneously. Respiration was recorded by a simple method which has been used by one of us for several years [Gunn, 1930]. The recording is done by a frontally writing lever such as is commonly used for recording the movements of smooth muscle, and a thread, attached to a selected point of the lever, is clipped to the fur of the animal's thorax or abdomen by means of a bulldog clip. This simple arrangement is very convenient for routine pharmacological investigation. It gives a straight line record, as does the blood-pressure manometer, instead of an arc, so that the time relations of heart beats and respirations can, if desired, be easily determined. No restricted alignment of the animal, in relation to the lever is necessary. The lever can be arranged so that it can be swung into apposition or rotated out of the way in a few moments. There are no tambours or rubber tubing to be renovated. With this arrangement inspiration is recorded by a downward movement of the lever.

A preliminary comparison of the three new compounds under these conditions showed that both phenylallylamine and phenylbutenylamine produced an increase in the rate of respiration, the latter being the more powerful stimulant, while diphenylethylamine had no such action. For

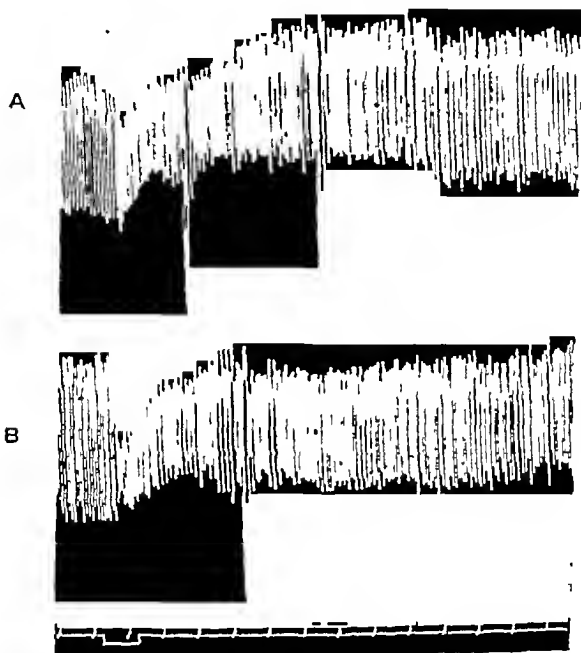


Fig. 1. Rabbit, urethane anaesthesia. Record of respiratory movements, inspiration downwards, showing similarity of respiratory stimulation produced by 10 mg. benzedrine (tracing A) and 6 mg. phenylbutenylamine (tracing B).

example, a rabbit received equal doses (3.4 mg./kg.) of the three compounds; phenylbutenylamine increased the respiration rate by 82%, phenylallylamine by 45%, while diphenylethylamine produced no change in the respiration rate.

As the stimulant action of phenylbutenylamine on the respiration was found to be so pronounced and constant that it might be of therapeutic value as a respiratory stimulant, further experiments were made to compare its activity in this respect with that of other respiratory stimulants, especially with benzedrine and lobeline. The respiratory stimulant action of benzedrine was first described by Alles & Prinzmetal [1933] and that of lobeline by Wieland & Mayer [1922].



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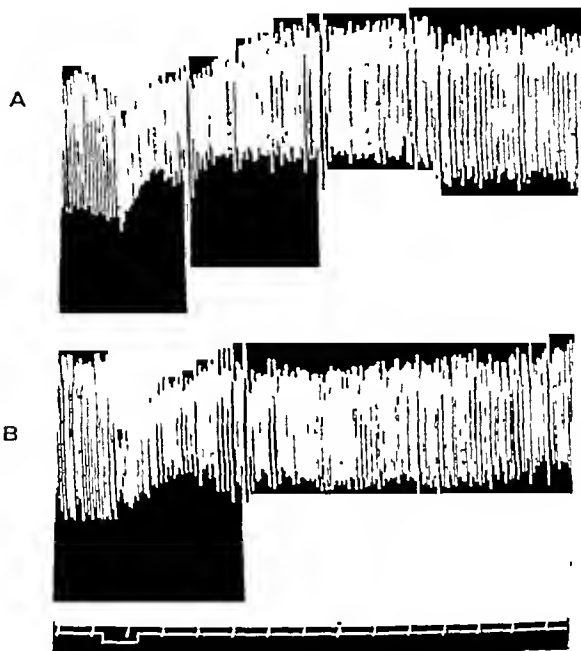


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15 min. Five minutes later (20 min. after the previous injection of phenylbutenylamine) the respiration rate being 60 per minute and the blood pressure 134 mm. Hg. lobeline (2 mg./kg.) was injected. This lowered the blood pressure to 90 mm. and produced a transient irregularity of respiration with some general muscular movements. One minute after injection, the respiration became regular at the rate of 66 per minute. In this experiment lobeline produced less stimulation of respiration than did phenylbutenylamine.

Since the respiratory depression induced by so large a dose of morphine subcutaneously administered, would persist for much longer than 2 hr., it

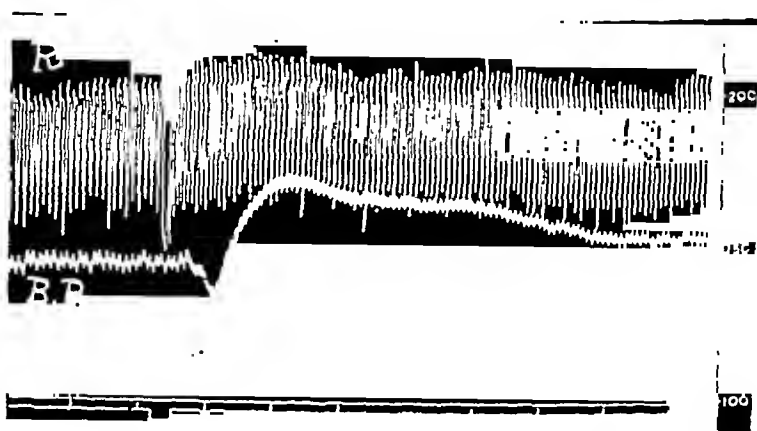


Fig. 2. Rabbit, morphine-ether anaesthesia, showing respiratory stimulation and pressor action of phenylbutenylamine 2 mg./kg.

is reasonable to conclude that phenylbutenylamine stimulates the respiration in a rabbit which has received a high narcotic dose of morphine.

Some experiments were done to compare the respiratory stimulant action of lobeline with that of phenylbutenylamine in rabbits anaesthetized with urethane (2 g./kg. subcutaneously), an example of which is illustrated in Fig. 3. Lobeline (0.25 mg./kg.) increased the respiration rate by a maximum of 50% but the rate returned to normal in 3 min. Phenylbutenylamine (0.75 mg./kg.) increased the respiration rate by a maximum of 54%; 3 min. after injection the rate was still 30% above normal and the normal rate was not reached until 10 min. later. The dose of phenylbutenylamine given was three times that of lobeline. These relative doses of the two substances were selected for comparison because Chakravarti [1939] had found that the equivalent dose for



A graphic record of respiration in an unanaesthetized animal could be obtained in the decerebrate cat. An illustration of the effect of phenylbutenylamine on respiration and blood pressure is given in Fig. 4. A dose of 2 mg./kg. increased the respiration rate from 12, to a maximum of 18 per minute, and, 15 min. later, the respiration rate was still 16 per minute.

So far as the respiration rate is concerned, therefore, all experiments agree in showing that phenylbutenylamine is an effective and consistent respiratory stimulant. This has been found in unanaesthetized mice, guinea-pigs and rabbits, in the decerebrate cat, and in rabbits anaesthetized by urethane or narcotized by morphine. In a few experiments which can only be regarded as preliminary, phenylbutenylamine has proved at least as effective a respiratory stimulant as benzedrine or lobeline. It is well known that the effectiveness of analeptics varies with the depressant which they are employed to antagonize and a much wider basis of experiment would be necessary for a quantitative comparison of phenylbutenylamine with other respiratory stimulants. Experiments to this end are in progress.

*Primary apnoea.* In a recent study of the action of benzedrine on the respiration, Chistoni & Beccari [1940] conclude that the respiratory stimulation produced by this compound is due to two factors, a direct stimulant action on the bulbar respiratory centres and a stimulation of the chemoreceptors in the carotid sinuses. We have as yet made no similar analysis of the nature of the respiratory stimulation produced by phenylbutenylamine. They draw attention, however, to the difference between benzedrine and adrenaline in regard to the initial transient apnoea. The fact that adrenaline may produce such an apnoea was pointed out by Oliver and Schäfer and the phenomenon has been extensively studied by many workers and recently reviewed by Marri & Hauss [1939]. Chistoni & Beccari failed to obtain apnoea with benzedrine in the dog anaesthetized by chloralose, but observed partial apnoea when barbitol was used as an anaesthetic, in which case the apnoea was ascribed to a reflex stimulation of the carotid sinuses by the rise of blood pressure. Various explanations have been given of adrenaline-apnoea but it has been very commonly held that it is secondary to the concomitant hypertension.

We have occasionally (three out of ten experiments) encountered apnoea with phenylbutenylamine in rabbits anaesthetized by urethane, and, certainly with this compound, it is impossible to attribute the apnoea to general hypertension, because the apnoea may occur with an

went, showed that, in the spinal cat, the former has about  $1/800$  to  $1/400$  of the pressor activity of adrenaline and that its pressor action lasts approximately 8 times as long as that of adrenaline.

*Tachyphylaxis.* It has been observed by many workers and with several compounds related to adrenaline that, if an initial intravenous dose gives a certain rise of blood pressure, a second injection of the same dose may have a much feebler pressor effect, or no effect, or sometimes even a depressor effect. This reduction of pressor activity with repetition of injections (tachyphylaxis) has been observed especially with ephedrine and benzedrine, and a discussion of the problem in the case of benzedrine has recently been given by Chistoni & Beccari [1938]. The degree of tachyphylaxis depends upon the dose of the drug and the interval between the injections but, even if these factors are kept constant, marked variations are shown by different individuals even of the same species of animal.

In regard to tachyphylaxis, adrenaline shows a sharp contrast to ephedrine or benzedrine because (1) a second injection of adrenaline, even if administered shortly after the first, usually produces a higher rise of blood pressure than the first, and (2) in an animal in which a first injection of benzedrine has almost annulled the pressor effect of a second injection of benzedrine the pressor action of adrenaline may be still unimpaired.

The usual explanation given of tachyphylaxis, is that a compound which displays this phenomenon, once it has combined with the receptors upon which it acts, "occupies" those receptors for a time in such a way that they cannot take up further amounts of the compound.

Seeing that adrenaline still acts during this "occupation", it is inferred that it acts on different receptors from those occupied by the compound producing tachyphylaxis.

We have found that tachyphylaxis occurs in spinal cats with phenylbutenylamine, though, in our experience, to a less extent than with benzedrine. An interesting point, however, is that a first injection of benzedrine may annul the effect of a subsequent injection of phenylbutenylamine as completely as of benzedrine itself, as is shown in the following experiment (Fig. 7). In a spinal cat a first injection of benzedrine (2.5 mg./kg.) raised the blood pressure by 90 mm. Hg, (a) and 20 min. later, it was still 20 mm. above normal. Phenylbutenylamine (2.5 mg./kg.) was then injected (b) and produced a rise of pressure of 10 mm. lasting only 20 sec. Five minutes later the same dose of benzedrine was equally ineffective (c), though, after another 5 min. interval, adrenaline (5  $\mu$ g./kg.)

phenylbutenylamine was greater than that of phenylallylamine, while diphenylethylamine was usually depressor, occasionally feebly pressor, in any effective dose. The pressor effect of phenylbutenylamine in a rabbit anaesthetized by morphine-ether is shown in Fig. 1, in which a dose of 2 mg./kg. raised the pressure from 142 mm. Hg to 170 mm.

In the decerebrate cat, phenylbutenylamine produced a similar rise of pressure, often preceded by a transient fall (see Fig. 4).

Experiments on the spinal cat gave results analogous to those described above, showing that the pressor effect of phenylbutenylamine is

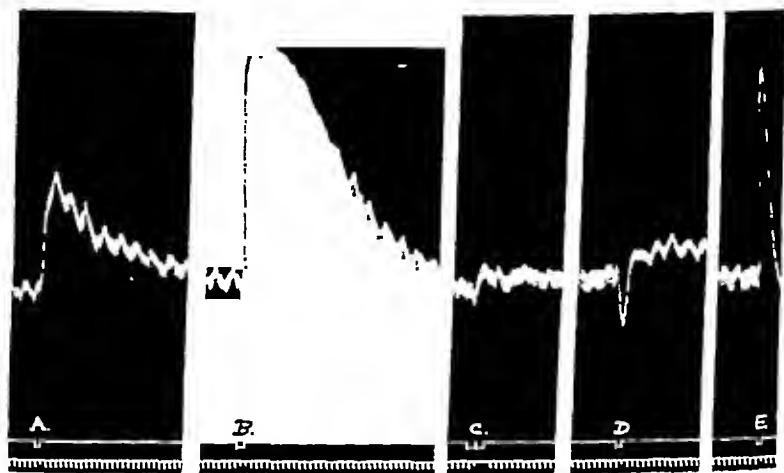


Fig. 6. Spinal cat, showing comparative effects on blood pressure of 3 mg. (A) and 10 mg. (B) phenylbutenylamine, of 3 mg. (C) and 10 mg. (D) diphenylethylamine, and of 10  $\mu$ g. (E) adrenaline.

greater than that of phenylallylamine while diphenylethylamine has very little effect on blood pressure in corresponding doses. A comparison of phenylbutenylamine, diphenylethylamine and adrenaline is shown in Fig. 6. A dose of 3 mg. of the first compound raised the blood pressure by 60 mm. (A), one of 10 mg. by 120 mm. (B), while corresponding doses of diphenylethylamine (C and D) produced only trifling effects. A subsequent injection of 10  $\mu$ g. of adrenaline increased the pressure by 104 mm. The absence of pressor effect of diphenylethylamine was not due to its being injected after phenylbutenylamine because the same result was obtained if the order of injections was reversed. The number of experiments done were not sufficient accurately to equate the pressor activities of phenylbutenylamine and adrenaline, but so far as they

*Peripheral sympathomimetic action*

It has been found by many workers that the actions of few compounds related to adrenaline reproduce the peripheral sympathomimetic effects on various organs containing smooth muscle with the same completeness and fidelity that are shown by adrenaline itself. Epstein *et al.* [1932] examined the actions of certain methoxyphenylethylamines and, from the responses given by a variety of organs, came to the conclusion that these compounds are sympathomimetic in the cat, but not in rodents.

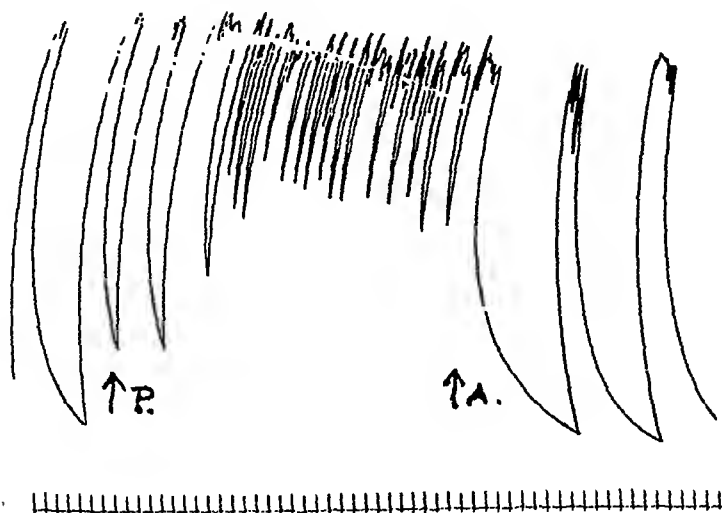


Fig. 8. Isolated uterus of non-pregnant cat, showing stimulation by phenylbutenylamine (1 in 25,000) and relaxation by adrenaline (1 in  $10^6$ ).

We have considered this question in a recent paper [Gunn & Gurd, 1940] and the discussion need not be traversed again. It may merely be recalled that phenylethylamine, cyclohexyl-ethylamine and the corresponding isopropylamine compounds do not always produce the same effect as adrenaline even on the same excised organ. For example, a guinea-pig's uterus which is inhibited by adrenaline may be stimulated by any one of these compounds. The actions of such compounds which are not strictly sympathomimetic do not admit of any known simple classification, such as "relaxation" or "contraction" of involuntary muscle as a whole, because the smooth muscle of one organ may contract, and that of another relax, under the influence of the same compound. Until some

raised the pressure by 90 mm. (*d*). Even after the lapse of 90 min., phenylbutenylamine raised the pressure by only 20 mm.

This experiment illustrates what was consistently found, namely, that benzedrine produces a high degree of tachyphylaxis not only to itself but also to phenylbutenylamine. The contrary, however, is not true to anything like the same extent, for phenylbutenylamine produces much less marked tachyphylaxis either to itself or to benzedrine. In an experiment, for example, in which the same doses were employed as in the previous experiment but the order of injections was reversed, a first injection of phenylbutenylamine (2.3 mg./kg.) raised the pressure by 96 mm. Hg and a subsequent injection of the same dose of benzedrine,

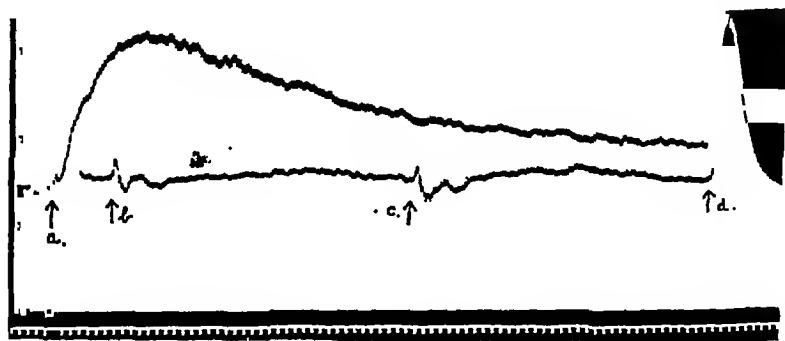


Fig. 7. Spinal cat, blood pressure showing "crossed tachyphylaxis" of phenylbutenylamine following benzedrine. At *a* and *c*, 2.5 mg./kg. benzedrine, at *b*, 2.5 mg./kg. phenylbutenylamine, at *d*, 5  $\mu$ g./kg. adrenaline.

given 5 min. later, raised the pressure by 110 mm. Subsequent injections of benzedrine produced a fall of pressure. We have found that, in contrast to what occurs with benzedrine, phenylbutenylamine can be given, in the spinal cat, in repeated doses at short intervals with little or no diminution of pressor activity, whereas the interpolation of a single similar dose of benzedrine at once diminishes or annuls the effect of subsequent injections either of benzedrine or of phenylbutenylamine. No simple explanation suggests itself for this difference in degree of "crossed tachyphylaxis" between the two compounds. It may be noted here also that, certainly with phenylbutenylamine and probably also with benzedrine, no corresponding phenomenon occurs with the respiratory stimulant action because little or no reduction in respiratory stimulation occurs with repeated equal doses, even when given at short intervals.

of adrenaline but about three times the duration. Both compounds, in concentration of 1 in 10,000 to 1 in 50,000 caused stimulation of the intestinal movements in the cat and relaxation in the rabbit. The same concentrations stimulated the uterus in the non-pregnant cat (Fig. 8), rabbit, guinea-pig and rat.

So far as excised organs are concerned, therefore, the responses of smooth muscle to these two compounds differ in many points from those to adrenaline, notably in the facts that the uterus of the non-pregnant cat, guinea-pig and rat and the intestine of the cat, all of which are inhibited by adrenaline, are stimulated by these compounds. In the spinal cat, however, with the organs *in situ*, the responses differ and are more definitely sympathomimetic. For example, in the experiment illustrated in Fig. 9, a record was taken of the blood pressure, intestinal movements and bronchial calibre (by Jackson's method). Phenylbutenylamine (2 mg./kg.) produced a rise of pressure, relaxation of the intestine, and dilatation of the bronchi. The same dose of benzedrine produced almost identical effects. Adrenaline produced effects qualitatively similar though of more brief duration. Dilatation of the bronchi in the cat and dog with benzedrine was first described by Alles & Prinzmetal [1933]. Diphenylethylamine, in concentrations of 1 in 5000 to 1 in 50,000 produced relaxation of the muscle of all excised organs with exception of the rabbit's uterus. As its effect on blood pressure is also mainly depressor, its action on smooth muscle can be regarded as generally depressant, with exception of the rabbit's uterus which is often stimulated by other substances which depress most other forms of smooth muscle.

### DISCUSSION

One object of the present investigation was to determine the effects of substitution of an unsaturated side-chain in phenylethylamine. Phenylallylamine, with the side-chain  $-\text{CH} : \text{CH} \cdot \text{CH}_2\text{NH}_2$ , is, in toxicity and general actions, almost indistinguishable from  $\beta$ -phenylethylamine, with the side-chain  $-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{NH}_2$ . Phenylbutenylamine, with the side-chain  $-\text{CH} : \text{CH} \cdot \text{CH}(\text{CH}_3)\text{NH}_2$ , very closely resembles, both in toxicity and general actions, benzedrine which possesses the side-chain  $-\text{CH}_2\text{CH}(\text{CH}_3)\text{NH}_2$ . So far as these compounds are concerned, therefore, the addition of an unsaturated carbon atom to the side-chain has remarkably little effect on physiological activity. Otherwise regarded, the group  $-\text{CH} : \text{CH}-$  is, in these compounds, almost equivalent to  $-\text{CH}_2-$ , a result which is not unexpected on theoretical considerations. On the other hand, phenylbutenylamine is not identical with benzedrine



satisfactory explanation of these differences in response is forthcoming, there seems nothing to be done beyond recording the experimental findings.

We have examined the actions of the three compounds here under consideration on the isolated heart (cat and rabbit), isolated intestine

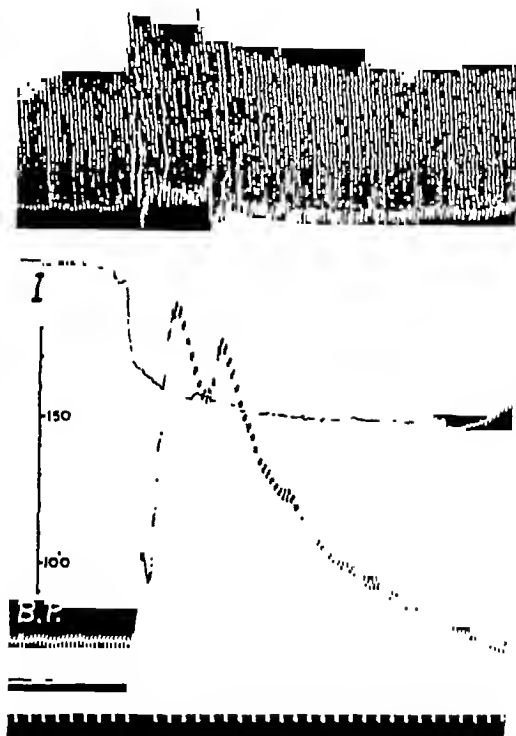


Fig. 9. Spinal cat, records of bronchial calibre, intestinal movements (I.) and blood pressure (B.P.). Phenylbutenylamine (2 mg./kg.) produced dilatation of the bronchi, relaxation of intestinal muscle, and rise of blood pressure, all sympathomimetic effects.

(cat, rabbit and guinea-pig) and isolated uterus (cat, rabbit, guinea-pig and rat). So far as phenylallylamine and phenylbutenylamine are concerned, their actions on these organs resemble so closely those of phenylethylamine and benzedrine that it is scarcely necessary to submit detailed descriptions or illustrations. Both compounds produce the characteristic augmentation and acceleration of the isolated cat's heart, phenylallylamine having an intensity of action of the order of 1/300

The phenylallylamine used in these experiments was synthesized by Dr C. J. Virden in the Pharmacological Laboratory, Oxford, several years ago. A supply of the other two compounds was obtained from Messrs Imperial Chemical Industries, for which courtesy we wish to record our indebtedness to them.

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in its actions, the main difference being in its action on the central nervous system upon which it seems to act lower down than benzedrine. Probably a more detailed investigation would reveal other minor differences in action, as has been the almost universal experience in comparing members of other homologous series.

Diphenylethylamine, with the side-chain  $-\text{CH}_2\text{CH}(\text{C}_6\text{H}_5)\text{NH}_2$ , differs widely from benzedrine, with the side-chain  $-\text{CH}_2\text{CH}(\text{CH}_3)\text{NH}_2$ , in its actions on the central nervous system, on blood pressure and on smooth muscle in various organs. The substitution in this position of a phenyl for a methyl group, therefore, completely alters the physiological action.

As phenylbutenylamine proved to be so reliable a respiratory stimulant under a variety of conditions that it might be of therapeutic interest, its actions have been described in greater detail than those of the other two compounds.

#### SUMMARY

1. Phenylallylamine is, in its physiological activities, hardly distinguishable from phenylethylamine.

2. Phenylbutenylamine very closely resembles benzedrine in toxicity, in action on blood pressure and on smooth muscle in various organs. It differs somewhat from benzedrine in action on the central nervous system, having relatively less stimulating action on the motor cortex. It is a reliable respiratory stimulant under a variety of conditions, comparing favourably in this respect with either benzedrine or lobeline.

3. Neither phenylallylamine nor phenylbutenylamine are strictly sympathomimetic in action. The latter, for example, stimulates the isolated uterus in the rat, guinea-pig and non-pregnant cat, when these are inhibited by adrenaline. This compound has about  $1/800$  to  $1/400$  of the pressor activity of adrenaline but the pressor action lasts about 8 times as long.

4. So far as these two compounds are concerned, the lengthening of the side-chain by introduction of two unsaturated carbon atoms has remarkably little effect on physiological activity, the substitution of the group  $(-\text{CH} : \text{CH}-)$  being equivalent to  $(-\text{CH}_2-)$ .

5. Diphenylethylamine differs widely from benzedrine in its actions especially in having a mainly depressant action both on the central nervous system and on smooth muscle. The substitution of a phenyl for a methyl group in the  $\alpha$ -position in phenylethylamine has, therefore, a profound effect in altering the quality of physiological action.

A mercury valve was attached to the carotid cannula by a side tube, so that a rise of blood pressure could be prevented, and the pressure maintained at a constant level. The side tube was clipped off when the valve was not required.

Intracranial pressure was gradually raised to a level averaging 50 mm. Hg above the systemic blood pressure, and heart rate and blood pressure recorded. Approximately 30 sec. was taken in raising the intracranial pressure, which was maintained for 5-20 min. These experiments were then repeated with the mercury valve open, so that no rise of blood pressure occurred when the intracranial pressure was raised. In one experiment, the pressure in the cisterna magna was recorded, and it was found that the pressure in the cisterna did not rise to the level recorded on the syphygmomanometer dial, being approximately 10 mm. Hg lower.

### RESULTS

Ten experiments were carried out, and in each case a rise in the intracranial pressure was followed by a rapid rise of blood pressure and cardiac slowing. When the blood pressure was maintained at a constant level, the decrease in cardiac rate was nearly as great as when the blood pressure was allowed to rise.

The average rise of blood pressure, when the mercury valve was not in use, was from 140 mm. Hg to 195 mm. Hg, and the pulse rate fell from 190 to 107 per minute. The average intracranial pressure as recorded by the syphygmomanometer dial was 200 mm. Hg.

Using the mercury valve, the blood pressure did not rise more than 5 mm. Hg from 132 to 137 mm. Hg, but the heart slowed from 182 to 113 per minute.

Fig. 1 shows the results obtained in a typical experiment. The cardiac slowing comes on when the intracranial pressure exceeds the systemic pressure, but is maintained even when the blood pressure rises above the intracranial pressure.

### DISCUSSION

These experiments show that the cardiac slowing associated with raised intracranial pressure is independent of the rise of blood pressure. The degree of slowing when the pressure is kept constant is less than obtained in the controls, because there is a loss of blood via the mercury valve when the intracranial pressure is raised. This haemorrhage amounts to some 40 c.c. of blood, and such a loss would cause a cardiac acceleration.

The onset of the cardiac slowing was similar in both sets of experiments, coming on when the intracranial pressure exceeded the systemic blood

## THE RELATION OF HEART RATE TO INTRACRANIAL PRESSURE

By O. G. EDHOLM<sup>1</sup>

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*(Received 17 May 1940)*

A RISE in intracranial pressure above the level of the systemic blood pressure is followed by a rise of blood pressure and a slowing of the heart [Cushing, 1902]. The rise in blood pressure is independent of the vagus [Cushing, 1902], and of the carotid sinus [Guernsey, Weisman & Scott, 1933]. The cardiac slowing is not due to carotid sinus impulses [Heymans, 1928], but is abolished by section of the vagus [Cushing, 1902].

Although this evidence supports the view that the cardiac slowing is due to a direct stimulation of the vagus centre, the possibility that the slowing is secondary to the rise of blood pressure, acting via the cardio-aortic pressor receptors, has not been eliminated. This paper describes experiments to test this point.

### METHODS

Cats anaesthetized with ether followed by chloralose (0.056 g. per kg. body weight) were used for all experiments. Blood pressure was recorded by a mercury manometer connected to a cannula in the right common carotid artery. A Hürthle manometer connected to a cannula in the right femoral artery recorded the heart rate. The skull was trephined over the left parietal region, the opening being  $\frac{3}{4}$  in. in diameter. The dura was incised and a close fitting rubber bung, with a glass tube passing through, was inserted into the trephine opening. The glass tube was connected to a flask containing Ringer's solution at 38° C. The pump and recorder of a syphygmomanometer were attached to the flask. By pumping in the Ringer's solution the intracranial pressure could be raised over 200 mm. Hg and maintained at this level for 20 min.

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intracranial pressure. This was found by both Cushing (1902) and Eyster, Burrows & Essick (1909).

Pressor receptors cannot be responsible for the cardiac slowing, and it is concluded that the raised intracranial pressure stimulates the vagus centre directly.

#### SUMMARY

1. The cardiac slowing associated with raised intracranial pressure has been investigated in cats.

2. Preventing the rise of blood pressure produced by a raised intracranial pressure does not abolish the cardiac slowing.

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 Eyster, J. A. E., Burrows, M. T. & Essick, C. R. [1909]. *J. exp. Med.* 11, 489.  
 Guernsey, M., Weisman, S. A. & Scott, F. H. [1933]. *Arch. intern. Med.* 52, 306.  
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pressure level. At this point there must be an anaemia of the medulla, but since the cardiac slowing persists after the blood pressure has risen above the intracranial pressure, it is unlikely that anaemia is the cause.

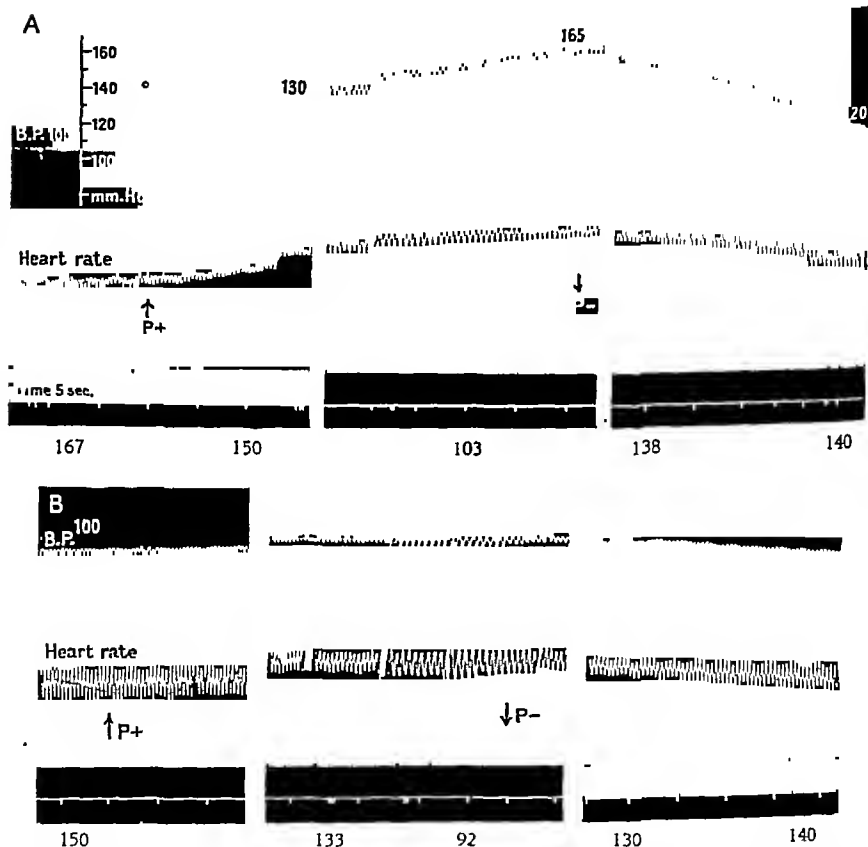


Fig. 1. Record of blood pressure and heart rate. At points marked *P+* intracranial pressure raised to 170 mm. Hg, at *P-* the pressure is lowered. The intracranial pressure changed over a period of 35 sec. To obtain the figure for the pressure exerted on the medulla, 10 mm. Hg should be deducted (see text). In the lower tracing, the mercury valve is in use, keeping the blood pressure constant. The heart rate per minute is given underneath the tracings.

Although the intracranial pressure, as recorded by the syphygmomanometer, was in many cases higher than the systemic pressure reached, the experiment in which the pressure in the cisterna magna was measured showed that the pressure here was 10 mm. Hg lower, and if this correction is used, in all cases the systemic blood pressure is found to rise above the

of the endolymph pipette, which is thus introduced into the scala media without any danger of picking up cerebro-spinal fluid on the way.

The depth to which the pipette is advanced is controlled by the appearance of fluid therein, which must be endolymph, provided that the suction apparatus keeps the field clear of all cerebro-spinal fluid.

Serial sections of the cochlea have been made in six animals and exclude the perforation of Reissner's membrane by the pipette as a likely complication.

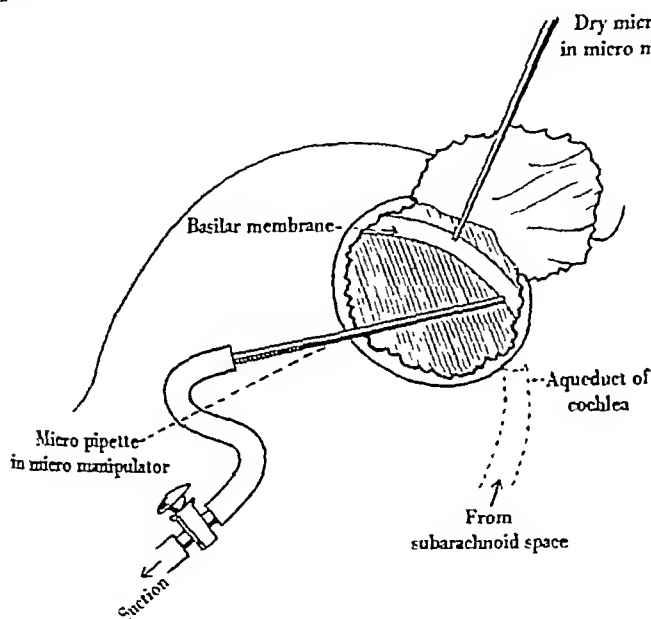


Fig. 1.

A sample of cerebro-spinal fluid is then collected from the cisterna magna. For this purpose the posterior atlanto-occipital membrane is exposed by dissection and punctured under direct vision with a dry glass capillary. Finally, a sample of blood is collected from the carotid artery into a glass tube containing a trace of heparin as an anti-coagulant.

*Difficulties.* In all, some thirty experiments were performed. At first a good deal of variation in the osmotic pressure values was met with. The factors chiefly concerned appeared to be depth of anaesthesia and prolongation of the operative procedure beyond  $1\frac{1}{2}$  hr.

With regard to the actual procedure of collection of the various fluids, no difficulty was encountered with blood, C.S.F., or perilymph, and once adequate speed of operation and control of anaesthesia was obtained the



OBSERVATIONS ON THE OSMOTIC PRESSURE  
OF THE ENDOLYMPHBY P. ALDRED,<sup>1</sup> C. S. HALLPIKE<sup>2</sup> AND A. LEDOUX<sup>3</sup>*From the Ferens Institute of Otology, Middlesex Hospital, and the  
Department of Physiology, University College, London**(Received 24 May 1940)*

ALTHOUGH the work of Guild [1927], Shambaugh [1932] and others has provided valuable circumstantial evidence that the metabolic processes of Corti's organ are effected in the mammal through the medium of the endolymph, no investigations have yet been carried out upon the chemical or physical attributes of this fluid on account chiefly of the technical difficulty of its collection.

Fig. 1 illustrates a method whereby it has proved possible in the cat to obtain endolymph samples large enough for the thermo-electric estimation of its total osmotic pressure.

The round window is exposed under light nembutal anaesthesia and a sample of perilymph is first collected by piercing the membrane with a dry glass capillary carried on a micro-manipulator. The membrane is then detached from its bony insertion with a needle and displaced upwards, exposing clearly the inferior aspect of the basilar membrane in its basal turn. A micro-pipette, the point of which is constructed to be somewhat smaller than the width of the basilar membrane, is then arranged by means of the micro-manipulator with its point close to the membrane and at right angles thereto.

Meanwhile, the field is filled with cerebro-spinal fluid flowing into the internal ear via the cochlear aqueduct. A second suction pipette is arranged with its point close to the opening of the aqueduct.

By the opening of a tap it is possible in this way to clear the field of cerebro-spinal fluid and leave the basilar membrane dry for the entry

<sup>1</sup> Working with a grant from the Royal Society.

<sup>2</sup> Foulerton Research Fellow, Royal Society.

<sup>3</sup> Research Fellow of the Belgian Universities Foundation.

theory of its origin as a secretion by the choroidal plexus, as fully discussed by Flexner in a recent review [1934].

(2) The osmotic pressure of the perilymph exceeded that of the c.s.f. in every case but one, in which equal values were obtained. The average values were as follows: c.s.f. 1.017; perilymph 1.047.

(3) In three of the six cases the osmotic pressure of the endolymph was found slightly to exceed that of the perilymph. In the other three cases this result was reversed. The average pressure of the endolymph exceeded that of the perilymph by 0.01% NaCl. On account of the difficulties described in the collection of the endolymph no significance can be attached to this difference. The measurements have therefore been taken as indicating that endolymph and perilymph are virtually iso-osmotic at a level equivalent to about 1.052% NaCl. The osmotic pressure of the c.s.f. was equivalent to 1.017% NaCl.

### DISCUSSION

The anatomical and osmotic conditions within the labyrinth may now be summarized in the form of a diagram (Fig. 2). The endolymph sac is filled by a secretion of the stria vascularis. This drains into the saccus endolymphaticus, and through its walls into the loose perisaccular connective tissue. Thereafter it is probably absorbed into small venous tributaries of the lateral sinus.

The perilymphatic space containing perilymph is bounded by bone with the elastic membrane of the round window adjoining the opening of the narrow cochlear aqueduct which leads from the subarachnoid space of the posterior fossa.

The present findings appear to be most readily explicable upon the hypothesis that the osmotic pressure of the endolymph at its source is high, and that a correspondingly high osmotic pressure of the perilymph is maintained by diffusion through the thin membrane separating the two fluids.

According to this hypothesis the cavity of the bony labyrinth may be regarded as a virtually closed space in which the contained fluids, both endolymph and perilymph, are in effect derived entirely from the endolymph. The presence of the cochlear aqueduct makes it impossible of course to regard the labyrinth cavity as being in fact an entirely closed space, and to justify the view put forward it is necessary to assume that the flow of endolymph is rapid relative to the rate of the c.s.f.—perilymph exchange through the cochlear aqueduct.

otic pressure values obtained for these fluids were considered on unical grounds to be trustworthy.

The case of the endolymph was more difficult, since, following the ringing of the round window membrane, a fairly free flow of c.s.f. lys occurred, and any failure of the suction technique resulted in the mixture with the endolymph specimen of varying amounts of c.s.f. Measurements were made of the total osmotic pressure of the blood, F., perilymph and endolymph. Baldes' [1934] modification of Hill's thermo-electric method was employed, and several pairs of especially all thermo-couple loops were constructed by Mr J. L. Parkinson, to whom our best thanks are due. These were used alike for all of the fluids.

Errors due to evaporation of the fluid samples were minimized by using pipettes of approximately standard dimensions, and so far as possible the samples were of constant After use, the pipettes were at once fixed with plasticine in the vertical position on inside of a narrow bottle, with their tips under mercury and so maintained until the measurements were made. This was effective in preventing the fluid samples from rising the wider portion of the pipettes where evaporation would be greatly accelerated.

All tests were carried out in an atmosphere in the testing chamber oxygen and 5% carbon dioxide.

The results to be described were obtained with a final series of six trials, in each of which the total procedure was completed without unical fault within  $1\frac{1}{2}$  hr.

### RESULTS

The figures given represent average values of a number of estimations, usually from 3 to 6. They express the total osmotic pressure in equivalent percentages of NaCl (g. NaCl per 100 g.  $H_2O$ ) and are subject to a probable error of  $\pm 0.005\%$ .

	Blood	c.s.f.	Perilymph	Endolymph
1	0.992	1.025	1.037	1.033
2	0.990	1.013	1.013	1.048
3	1.020	1.036	1.044	1.100
4	0.980	1.005	1.054	1.073
5	0.990	0.994	1.038	1.024
6	0.994	1.029	1.093	1.073
Average	0.994	1.017	1.046	1.058

These results may be summarized as follows:

(1) The osmotic pressure of the c.s.f. was in every case found to exceed that of the blood. The average values were as follows: blood 0.994; F. 1.017.

It appears justifiable to regard this difference as representing a well-defined gain of free energy by the c.s.f. in accordance with the

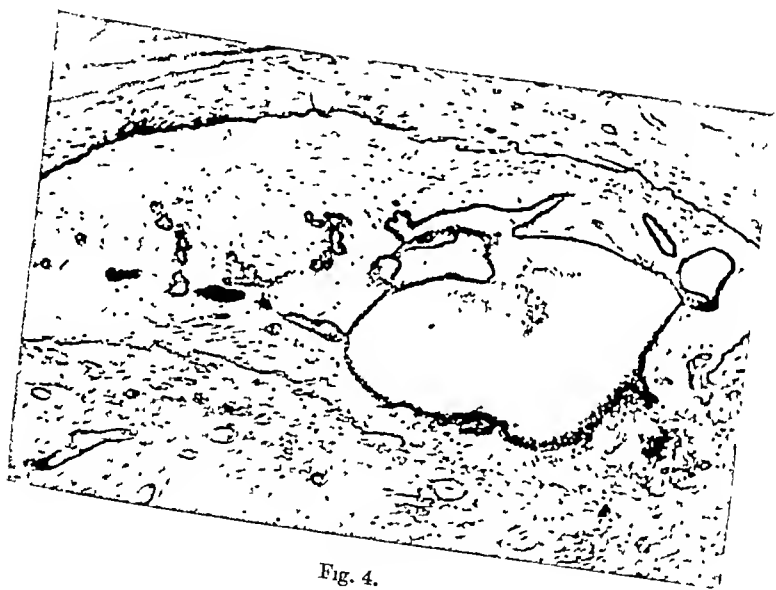


Fig. 4.

No data are available upon the rate of flow of the endolymph, although in view of its probable function, its independent pathway, and the bulk and vascularity of the stria vascularis this may well be considerable.

With regard to the rate of exchange of the c.s.f. and perilymph through the cochlear aqueduct it is possible to indicate the magnitude of certain limiting factors. The freedom of flow of fluid through the aqueduct in response to pressure changes in the subarachnoid spaces is dependent upon the bulk modulus of the bony labyrinth, the value of

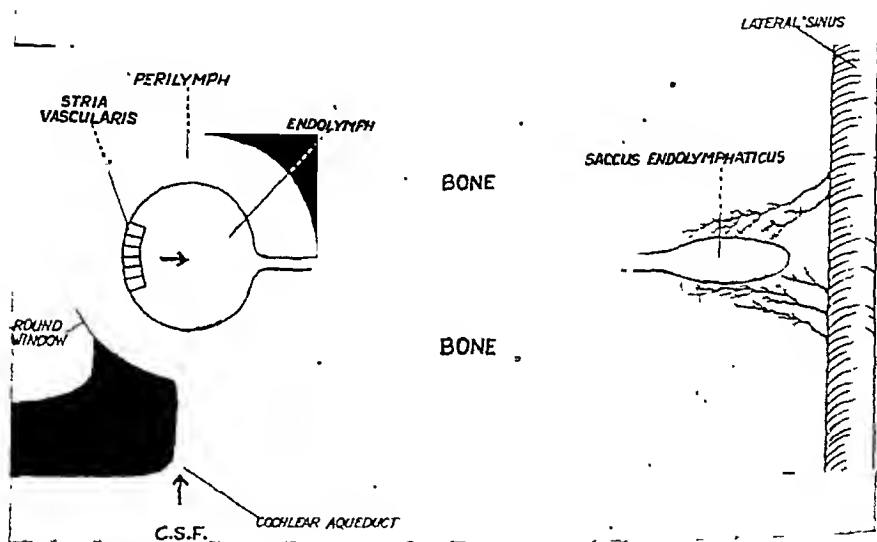


Fig. 2.

which in the absence of the round window membrane would in all probability be enormous, approximating to that of water itself and would virtually prohibit any fluid movement through the aqueduct.

The effectiveness of the round window membrane in reducing this value would depend in turn upon the elasticity of its fibres and upon its area relative to that of the rest of the labyrinthine wall. No information regarding the former is at present available. With regard to the latter, data on the human labyrinth were obtained by projecting serial histological sections at a convenient magnification and by measuring the total lengths in all the sections of the internal contours of the bony labyrinthine wall and of the round window membrane.



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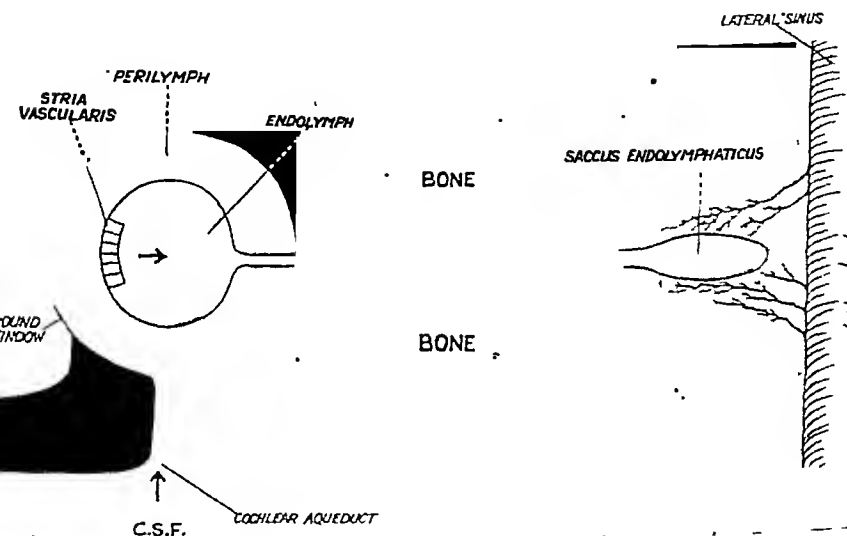


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For this purpose a recording map measuring device of the usual rotating wheel type was used. The ratio of these lengths, and accordingly of areas, for round window membrane and bony labyrinthine wall was found to be as 1 : 257, and the relative smallness of the membrane must accordingly be regarded as a considerable factor in limiting the flow of C.S.F. through the cochlear aqueduct. Certain questions concerning the ultimate fate of the endolymph are raised by the measurements of its osmotic pressure which have been described and consideration may be given to certain structural aspects of the saccus endolymphaticus and

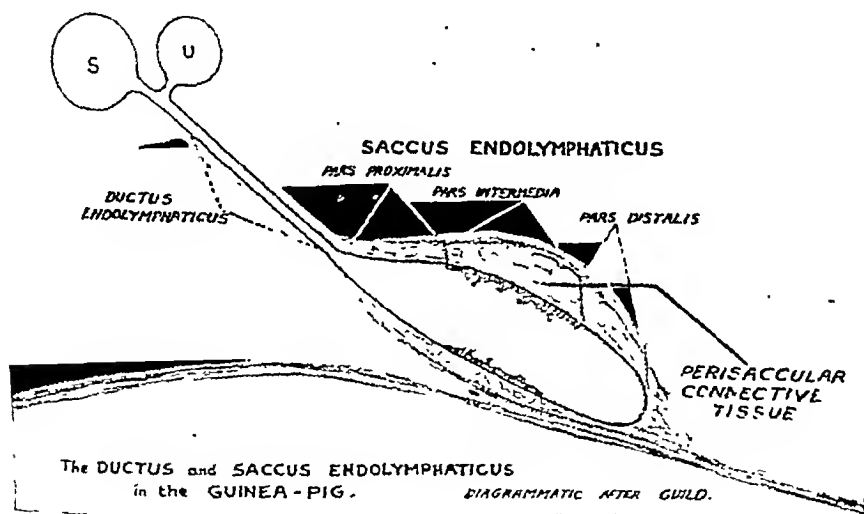


Fig. 3.

its surroundings. Fig. 3 is a diagrammatic representation of the mammalian ductus and saccus endolymphaticus according to Guild [1927]. The narrow tube lined by flattened epithelium is the ductus and all of the wider distal part is the saccus, which Guild divides into three parts, proximal, intermediate and distal. The proximal and distal parts are lined by flattened epithelium and the intermediate part by a low columnar epithelium with recesses and papillary ingrowths. Surrounding this "pars intermedia" is a very loose vascular connective tissue.

Fig. 4 shows the normal structure of the pars intermedia of the human saccus endolymphaticus with its surrounding loose perisaccular connective tissue containing the radicles of the adjacent lateral sinus.





Fig. 5A.



Fig. 5B.

## SUMMARY

A method is described of collecting small samples (5-10 cu. mm.) of endolymph in the cat.

The results are given of a short series of thermo-electric estimations of the total osmotic pressure of the blood, cerebro-spinal fluid, perilymph and endolymph.

The values obtained, expressed as equivalent percentages of NaCl (g. NaCl per 100 g.  $H_2O$ ), were respectively:

0.994, 1.017, 1.046, and 1.058.

The findings are considered to support the view of a secretory origin of both cerebro-spinal fluid and of the labyrinthine fluids, and to provide some indication of the relative rates of the flow of endolymph and of the cerebro-spinal fluid-perilymph exchange through the cochlear aqueduct.

A suggestion is made concerning the mechanism of the final disposal of the endolymph.

Acknowledgement is made to the Medical Research Council for the provision of an assistant to one of us (C. S. H.).

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- Baldes, E. J. [1934]. *J. Sci. Instrum.* 11, 223.  
Flexner, L. B. [1934]. *Physiol. Rev.* 14, 161.  
Guild, S. R. [1927]. *Amer. J. Anat.* 39, 57.  
Shambaugh, G. E. [1932]. *Special Cytology*, 3, 1332. New York: P. B. Hoeber.

By means of the Prussian blue granule technique Guild was able to show that the endolymph is absorbed through the wall of the pars intermedia into this loose perisaccular connective tissue. The passage of the endolymph through the wall of the saccus endolymphaticus may thus be regarded as a fact based upon observation by Guild. It must be added that Guild himself made no suggestion as to the further disposal of the endolymph, and the assumption of its final absorption into the radicles of the lateral sinus is arrived at by a process of exclusion.

The value for the osmotic pressure of the blood is given as equivalent to 0.994, and of the endolymph to 1.052 g. NaCl per 100 g. H<sub>2</sub>O, representing pressures of 2892 and 3061 mm. Hg respectively.

The hydrostatic pressure within the labyrinth approximates to that of the c.s.f., while the pressure within the lateral sinus is approximately atmospheric. The maximum filtration pressure within the endolymphatic sac is thus unlikely to exceed some 10 mm. Hg, leaving a pressure balance of 160 mm. Hg to be overcome by the intervention of some unknown mechanism.

The nature of this mechanism can at present be only a matter for conjecture and must, it would seem, involve two distinct processes. In the first of these, "the excretion stage", endolymph is transferred by the excretory activity of the epithelium of the pars intermedia into the perisaccular connective tissue which is then distended by water attracted to the osmotically stronger endolymph. This second process, "the dilution stage", has a dual effect; first, of increasing the perisaccular pressure by swelling of the perisaccular connective tissue, thus opposing the further excretion of endolymph from the saccus, and secondly, of so reducing the osmotic pressure of the excreted endolymph as to render possible its absorption into the blood stream.

This is followed by a further phase of endolymph excretion from the saccus.

This hypothetical mechanism of transfer of the endolymph from the lumen of the saccus to the blood stream is supported by observation to a limited extent by the wide variations in the bulk and consistency of the perisaccular connective tissue which are found to occur in normal material, particularly in the human subject.

Thus, Fig. 5A would correspond to the "excretion stage" with a moderate bulk of perisaccular connective tissue. In Fig. 5B (identical magnification) the dilution stage is far advanced with extreme distension of the meshes of the perisaccular connective tissue.

## SUMMARY

A method is described of collecting small samples (5-10 cu. mm.) of endolymph in the cat.

The results are given of a short series of thermo-electric estimations of the total osmotic pressure of the blood, cerebro-spinal fluid, perilymph and endolymph.

The values obtained, expressed as equivalent percentages of NaCl (g. NaCl per 100 g.  $H_2O$ ), were respectively:

0.994, 1.017, 1.046, and 1.058.

The findings are considered to support the view of a secretory origin of both cerebro-spinal fluid and of the labyrinthine fluids, and to provide some indication of the relative rates of the flow of endolymph and of the cerebro-spinal fluid-perilymph exchange through the cochlear aqueduct.

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## THE TIME COURSE OF EVOLUTION OF OXIDATIVE RECOVERY HEAT OF FROG'S MUSCLE

By D. K. HILL

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*(Received 28 May 1940)*

IN an earlier paper [D. K. Hill, 1940] a method was described for determining the time course of oxygen consumption of stimulated frog's muscle at 0° C. Comparison was made with the time course of oxidative recovery heat production under the same conditions and the results given in this paper were there quoted.

In the past there have been investigations at 0° C. of the initial heat production and of the negative phase in the anaerobic delayed heat, but never of the oxidative delayed heat. The reason is that the latter is evolved very slowly at such a low temperature and the thermopile-galvanometer system previously available did not possess the necessary combination of sensitivity and stability. The difficulty has now been overcome. The method and technique are due to A. V. Hill and have already been described in detail [A. V. Hill, 1937, 1938, 1939]. Accurate analysis of the galvanometer record over the complete period of recovery is greatly simplified by virtue of a certain property of the modern thermopiles. It is that the rate of fall of temperature at the hot junctions is almost exactly proportional to the temperature difference between the hot and the cold junctions: this means that a control heating curve has a logarithmic form. The application of this property in the analysis has been outlined by A. V. Hill [1939]. It will be seen that the oxidative delayed heat at 0° C. is not complete for about 30 min. and analysis for a period as long as this by the old method would be very laborious. By the new method the analysis can be completed in less than an hour.

## METHOD

The method has been described by A. V. Hill [1939]. The following points require mention:

(1) The solution for preliminary soaking of the muscles must have the same composition as that used in the experiments on oxygen consumption to ensure comparison under identical conditions. It consists of Ringer's solution containing 10 mg. P/100 c.c. in the form of phosphates at pH 7.2.

(2) The adequacy of supply of oxygen to the innermost layers of the muscle must be considered. The thickness of muscle lying on the thermopile is never more than 1 mm. The resting rate of oxygen consumption at 0° C. is  $10^{-4}$  c.c./c.c.  $\times$  min. Krogh's diffusion constant may be taken as  $1.1 \times 10^{-5}$  at 0° C. The concentration of oxygen at a depth of 1 mm. in the muscle is (by calculation) 29.5 mm.<sup>3</sup>/c.c. [see A. V. Hill, 1928]. With a 12 sec. tetanus the total quantity of oxygen required for complete recovery is 26 mm.<sup>3</sup>/c.c. of muscle. There is thus adequate supply. In two experiments the durations of tetanus were 18 and 24 sec. respectively and there is thus some possibility of lack of oxygen in the innermost layers of muscle. However, by reference to the previous paper [D. K. Hill, 1940] it will be seen that the oxygen consumption occurs so slowly after activity as to allow ample time for replenishment by diffusion!

(3) The duration of tetanus was varied from 2 to 24 sec. for different experiments. It will be seen that the time course of recovery heat production is nearly independent of stimulus duration over this range so that for purposes of comparison with the time course of oxygen consumption the duration need not be exactly specified.

## RESULTS

*The time course of delayed heat production at pH 7.2*

The results are given in Table I. The total quantity of delayed heat (anaerobic plus oxidative) is given at one minute intervals up to 30 min. for ten different experiments. The figures for the last six experiments have been added together. The average duration of tetanus for this group is 11 sec. which is approximately the duration of stimulus employed in the oxygen consumption experiments. In order to make a comparison of the time courses of oxidative delayed heat and oxygen consumption the former has to be obtained by subtracting from the total delayed heat that part of the delayed heat which is still present in the absence of oxygen. It was shown by Hartree [1932] that this is a justifiable pro-

TABLE I. pII 7.2

	Time (min.)																														Duration of stimulus sec.	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29		30
I	0	2	11	23	36	46	53	60	64	71	75	80	83	87	89	91	93	94	95	97	97	98	99	99	99	99	99	100	100	100	100	100
II	0	4	14	22	30	38	45	51	57	62	67	71	75	79	83	85	88	90	91	93	95	96	97	98	99	99	100	100	100	100	100	100
III	0	0	19	28	35	43	49	55	60	65	70	74	78	81	83	86	88	90	91	93	94	95	96	97	97	98	98	99	99	100	100	100
IV	0	6	16	25	32	40	46	53	58	63	68	72	76	79	81	84	86	88	89	91	93	94	95	96	97	98	98	99	99	100	100	100
V	0	5	13	22	30	36	43	48	54	59	63	67	70	74	76	79	82	85	86	87	89	91	92	93	94	95	96	97	98	99	100	100
VI	0	5	13	22	30	36	43	48	54	59	63	67	70	74	76	79	82	85	86	87	89	91	92	93	94	95	96	97	98	99	100	100
VII	0	5	13	22	30	36	43	48	54	59	63	67	70	74	76	79	82	85	86	87	89	91	92	93	94	95	96	97	98	99	100	100
VIII	0	8	17	25	33	40	46	52	56	61	65	69	72	75	77	80	83	85	86	87	89	91	92	93	94	95	96	97	98	99	100	100
IX	0	0	21	31	40	47	54	59	64	69	72	75	78	81	83	85	87	89	91	92	93	94	95	96	96	97	98	98	99	99	100	100
X	0	8	18	26	35	42	47	53	59	63	67	70	73	76	79	81	83	85	87	89	90	91	92	93	94	95	96	97	98	99	100	100
Sum V-X	0	05	148	108	237	276	308	341	370	393	415	430	451	467	483	499	514	522	528	539	549	555	561	566	572	578	583	589	590	590	593	593
÷ 6	0	7	16	25	33	40	46	51	57	62	66	69	72	76	78	81	83	86	87	88	90	92	93	94	94	95	96	97	98	98	98	99
AN.D.H.	0	3	6	8	10	11	12	12	13	14	15	15	15	15	16	16	17	17	17	17	18	18	18	18	19	19	19	19	19	19	19	19
O <sub>2</sub> .D.H.	0	4	10	17	23	29	34	39	44	48	51	54	57	61	62	65	66	69	70	71	72	74	75	76	76	77	78	79	79	79	80	80
O <sub>2</sub> .D.H.	0	5	13	21	29	36	43	49	55	59	64	68	71	76	78	81	83	86	88	89	90	93	94	95	94	95	96	98	99	99	100	100

AN.D.H. = Anaerobic delayed heat. O<sub>2</sub>.D.H. = Oxidative delayed heat.

AN.D.II. — Anaerobic delayed heat. O<sub>2</sub> D.II. — Oxidative delayed heat.TABLE II.  $pH$  0

	1	2	3	4	5	6	7	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	Duration of stimulus
I	1	6	11	17	23	28	33	37	40	54	61	68	74	78	83	87	89	93	93	95	95	97	98	98	98	99	99	99	100	6
II	0	6	11	16	21	26	30	35	43	50	56	63	68	73	78	81	84	87	90	92	94	95	97	98	99	99	100	100	100	12
AN.D.H.	-2	-1	2	2	2	3	3	3	3	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	12
O <sub>2</sub> .D.H.	0	7	9	14	19	23	27	32	40	46	52	59	64	68	73	76	79	82	85	87	89	91	92	93	94	94	95	95	95	12
Same to	0	7	9	15	20	24	28	34	42	49	55	62	67	72	77	80	83	86	89	92	94	95	97	98	99	99	100	100	100	12
max. 100																														

AN.D.H. = Anaerobic delayed heat. O<sub>2</sub>.D.H. = Oxidative delayed heat.

cedure: he found that the early stages of the delayed heat at 17° C. are identical in the presence of oxygen and in the earliest runs of a series in the absence of oxygen. Included in Table I, therefore, is a column giving the figures for the earliest runs of a series in nitrogen. The figures are expressed as percentage of the initial heat. The sum of the six delayed heat experiments is brought to a final maximum of 100. The total delayed heat is very nearly equal to the initial heat [A. V. Hill, 1939] so the latter may be taken as 100 and the figures for the anaerobic

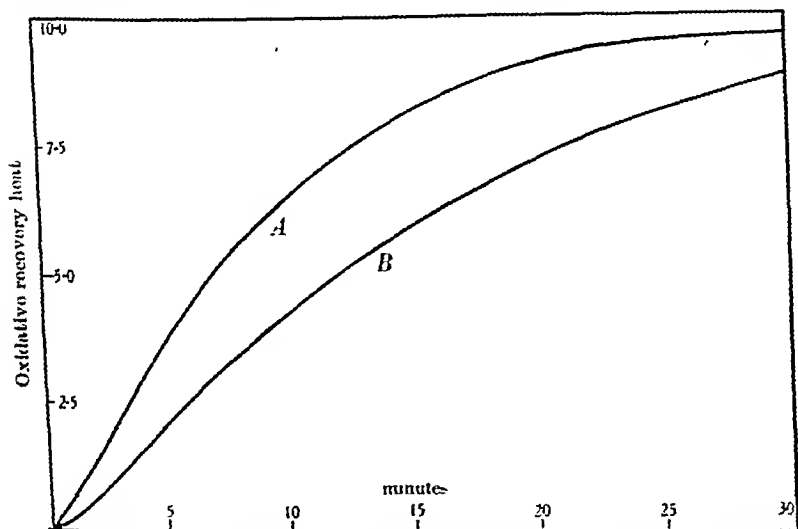


Fig. 1. Oxidative recovery heat following a 12 sec. tetanus at 0° C. A, pH 7.2; B, pH 6.2.

delayed heat at the corresponding times subtracted. The result gives the time course of the oxidative delayed heat (see Fig. 1). The determination of the anaerobic delayed heat is the subject of a later paper.

#### *The time course of delayed heat production at pH 6*

Oxygen consumption following a tetanus is considerably slowed by lowering the pH of the medium used for the preliminary soaking [D. K. Hill, 1940]. It is desirable to determine the time course of oxidative delayed heat production of muscle at 0° C. and pH 6 for comparison with the time course of oxygen consumption under these conditions.

The solution for preliminary soaking was made identical with that used in the oxygen experiments, namely Ringer's solution containing 25 mg. P/100 c.c. in the form of phosphates at pH 6. The results are shown in Table II. Each series is the mean of three runs taken with one



pair of muscles. At this  $pH$  the time course of total heat production varies with the duration of tetanus to a greater extent than at  $pH$  7.2. For comparison with the time course of oxygen consumption, therefore, the duration of tetanus must be specified: it should be 12 sec. The time course of *oxidative* delayed heat for this duration of tetanus is derived, in the same way as at  $pH$  7.2, by subtraction of anaerobic heat from total heat (Table II and Fig. 1).

### DISCUSSION

This research was done for the specific purpose of obtaining a comparison of recovery heat and oxygen consumption under identical conditions and for that reason is deficient in one respect: the variation of the time course of heat production with duration of tetanus has not been fully explored. The results in Table I do not demonstrate clearly the trend of variation with change in stimulus duration and experiments should be done using much shorter and much longer durations. The observed changes in time course of oxygen consumption with the longer stimuli is consistent with Hartree's results and the existence of a maximum possible rate of oxygen consumption is also to be correlated with Hartree's discovery of a maximum possible rate of oxidative recovery heat production. It is probable that corresponding changes would be found in the recovery heat production at  $0^{\circ}C$ .

At present the time course of oxygen consumption cannot be investigated accurately with stimuli of less than about 5 sec. so that comparison with very short durations of tetanus (up to 1 sec.) is impossible. It would nevertheless be worth while exploring these lower reaches especially for the purpose of investigating further a point mentioned by Hartree, namely that with short durations of stimulus the times to maximum of the rates of production of anaerobic and oxidative heat vary in a similar manner as the duration of stimulus is changed. This acquires special significance in view of the conclusions of the next paper where it is shown that the early rapid phase of production of the anaerobic delayed heat must be attributed to the breakdown and not to the re-synthesis of phosphocreatine. It would seem probable that the breakdown products of phosphocreatine are responsible for the initiation of the oxidative recovery processes in muscle.

### SUMMARY

1. Owing to improvements in technique it is possible to determine the time course of oxidative recovery heat production of frog's muscle at  $0^{\circ}C$ .

2. Following a 12 sec. tetanus this heat production is half complete in 7 min. at pH 7.2 and in 12 min. at pH 6.

3. At pH 7.2 the duration of tetanus was varied from 2 to 24 sec. This is not a sufficiently wide range to bring out clearly the dependence upon stimulus duration and further experiments are required using shorter and longer durations.

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## THE ANAEROBIC RECOVERY HEAT PRODUCTION OF FROG'S MUSCLE AT 0° C.

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THE anaerobic recovery heat of frog's muscle has received considerable attention in the past [Hartree & Hill, 1922, 1923; Furusawa & Hartree, 1926; Hartree & Hill, 1928; Hartree, 1929; Blaschko, 1930; Cattell & Hartree, 1932; Hartree, 1932]. Two phases have been distinguished, an early transient negative phase of heat absorption followed by a more prolonged positive phase. The great variability in the results obtained has always been emphasized and it is the purpose of this paper to show that, in certain circumstances, this variability can be interpreted and controlled.

The experiments to be described were done for two reasons:

(1) As explained in the previous paper, it was desired to determine, at 0° C., the true oxidative delayed heat, i.e. the part of the delayed heat which is caused by oxidative processes. It was seen that this could be done by subtracting from the total delayed heat the anaerobic delayed heat of the earliest runs of a series in nitrogen. The latter had therefore to be determined under the same conditions. (2) In a later paper it will be seen that a study has been made of the carbon dioxide exchange of muscles with oxygen uptake inhibited. These heat experiments were done for purposes of comparison and correlation.

This research is, therefore, not a general survey of the subject, since one important variable, namely, duration of tetanus, has been fixed at 12 sec. as required for both the comparisons mentioned.

### METHOD

A general description of the apparatus, the procedure and the method of analysis has been given elsewhere [A. V. Hill, 1939]. Some details require mention.

Following the usual period of soaking at 0° C. in oxygenated Ringer's solution (with 10 mg. P/100 c.c. as phosphates at pH 7.2) the latter was removed and cylinder nitrogen, freed from traces of oxygen by passing over red-hot copper gauze, was passed rapidly through the thermopile chamber for half an hour. It can be shown that in this time about 99% of the oxygen originally present in the muscle will have escaped by diffusion and the remaining 1% will be consumed by the resting muscle in the same time. The nitrogen was passed slowly through the thermopile chamber throughout the period of the experiment. This precludes possibility of entry of air by convection.

*At pH 6.* Experiments with the muscles at pH 6 were done by adding phosphate buffer (10 mg. P/100 c.c.) to the mixture used for the preliminary soaking.

*Procedure for experiments with muscles poisoned with iodoacetic acid.* It has been shown by Mawson [1932] that muscles under the influence of iodoacetic acid are capable of more prolonged activity if they have been previously soaked in Na-l-Lactate. It appears that the lactate can be oxidized and the onset of rigor thereby delayed.

The muscles were first soaked in the usual way in Ringer's solution containing phosphate at pH 7.2. 50% racemic sodium lactate was then added to make the concentration  $\frac{1}{2}$ %. 15 min. later sufficient  $M/25$  sodium iodoacetate was added to give a concentration  $M/2500$  and the soaking continued for 1 hr. At the end of this time the mixture was withdrawn from the muscle chamber and nitrogen was passed to dispel the oxygen. Muscles treated in this way are capable of prolonged activity without developing signs of rigor.

*Stimulus.* For reasons already given, the duration of tetanus was 12 sec. In the experiments on the oxidative delayed heat it was the practice to give preliminary trial stimuli consisting of single condenser discharges in order to find the voltage for maximal response. In these experiments, however, no trial stimuli were given because it is important to record the heat production of the muscles following the earliest bouts of activity. Anaerobically the time course of heat production varies greatly with the amount of previous activity and it is only by omitting trial stimuli that a complete sequence can be observed.

*Osmotic changes.* There is one complicating factor which is not encountered in the experiments with oxygen present: it is found that there is always a permanent final positive deflection from the original base line. This is due to an increase in osmotic pressure of the muscle which causes condensation of water vapour on the muscle and consequent

liberation of heat [see Hill & Kupalov, 1930]. Cattell & Hartree [1932] avoided this complication by filling the muscle chamber with oxygen-free paraffin oil: condensation of water vapour is thus prevented. One disadvantage of doing this is that the rate of loss of heat from the muscle is increased with a corresponding decrease in accuracy: but more important is the possibility that the valuable property of exponential temperature decay would be sacrificed. This possibility has not yet been tested.

Approximate allowance has been made for the osmotic pressure change in these experiments by subtracting the final permanent base-shift from the whole of the deflection time curve from the beginning.

*Fatigue of the muscles.* The initial heat production diminishes with successive stimuli. The series was discontinued when the initial deflection had fallen to about one-quarter of its original value. Muscles which are grossly fatigued are liable to non-uniform contraction with the anomalous results discussed by A. V. Hill [1938]. When the muscles have not become unduly fatigued they can be completely restored by soaking in oxygenated Ringer's solution.

*Analysis of the records.* It is necessary to analyse the galvanometer records singly and so obtain a series from one pair of muscles. In the case of the oxidative delayed heat it was seen that an absence of variation from one run to the next permitted the summing of a number of runs to obtain a representative result. Anaerobically, however, a regular and marked variation is discernible and any averaging of results would be arbitrary and not representative. With a good pair of muscles at 0° C. it has been found possible to give as many as 15 tetani, each of 12 sec. and without intermediate soaking in Ringer's solution.

## RESULTS

*With lactic acid production prevented.* In Fig. 1 is shown the analysed results for a series of runs with muscles at pH 6. A similar series was obtained with muscles poisoned with iodoacetic acid. Lactic acid production cannot occur at pH 6 [Kerly & Ronzoni, 1933] or in the presence of iodoacetic acid. It is only under such "alactacid" conditions that any regular progressive change is found in successive runs with one pair of muscles. The heat production is entirely negative only in the earliest runs when the muscles are in very fresh condition and trial stimuli have been omitted.

Under "alactacid" conditions the heat production and the return of the galvanometer spot are absolutely complete in 10 min. after the

activity. This is in marked contrast to the behaviour with the muscles under normal conditions when the heat production is not complete for more than 30 min. and then tails off so slowly as to make uncertain the true duration of recovery. Stress has been laid on this early conclusion of the delayed heat production under "alactacid" conditions because it proves that the lactic acid formation really has been inhibited. For in a subsequent paper it will be shown that increase in acidity of a muscle which, in the later stages of recovery, runs parallel to the lactic acid production, is only about one-quarter complete in 10 min.

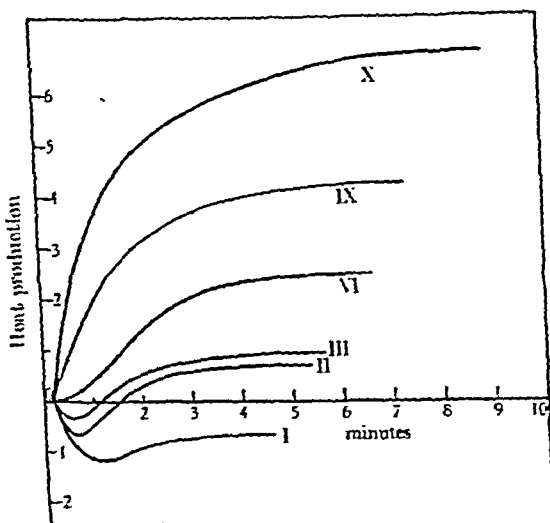


Fig. 1. Anaerobic delayed heat at 0° C. Series taken with muscles at pH 6. The Roman numerals refer to the position in the series. Ten runs were taken with this pair of muscles without intermediate soaking: four were not recorded. Total quantity of heat evolved is expressed as percentage of initial heat.

*Anaerobic delayed heat under normal conditions.* The results under "alactacid" conditions were given first because the number of concurrent processes associated with heat production is then smaller and the variation between successive runs correspondingly more ordered. When lactic acid production is permitted the early phases of heat production (occurring within 10 min. after the activity) are similar as regards magnitude and time course to a member of the series under "alactacid" conditions. Superimposed on this common phase is a much slower phase of heat production: the time course of its commencement cannot be accurately distinguished and its completion is indefinite and

certainly not before 30 min. (Fig. 2). As regards variation between successive runs it is found that the later slow phase does not undergo any obvious ordered variation although the early phase seems to show the same trend as under "alactacid" conditions. There is not yet much evidence in this respect.

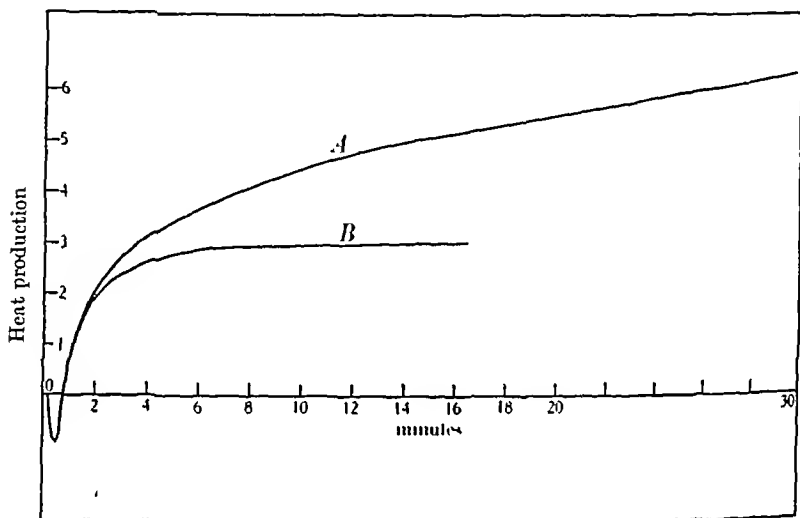


Fig. 2. *A*, anaerobic delayed heat at pH 7.2. *B*, a member of a series under "alactacid" conditions for comparison. Duration of tetanus 12 sec. The heat production is expressed as percentage of initial heat.

*Magnitude of anaerobic delayed heat.* (1) "Alactacid" conditions. In the longest series taken, the total heat varies from minus 2% of the initial heat for the first run to plus 15% for the tenth and last. (2) *Normal conditions.* The total heat for successive runs does not appear to vary in an ordered manner but there is not yet sufficient evidence in this respect. Some of the variation observed is due to inaccuracy in measuring the small second phase of heat production which tails off very slowly. For thirteen experiments the average total delayed heat was 19% of the initial heat, the values ranging from 6 to 29%.

#### DISCUSSION

Both phases of the anaerobic delayed heat were originally thought to accompany the coupled reaction between lactic acid production and phosphocreatine resynthesis, this coupling being more efficient in the early stages with the resultant heat absorption. It was unsatisfactory to assume such variation in coupling efficiency for Lundsgaard [1931]

had shown that there is a fixed relation between number of molecules of phosphocreatine resynthesized for 1 mol. of lactic acid produced.

Meyerhof, Schulz & Schuster [1937] provided a much better explanation for the existence of the two phases of heat production. It is based on the discovery that lactic acid is produced in two stages in each of which 1 mol. of phosphocreatine is resynthesized. Pyruvic acid is formed in the first stage and in the second stage is converted to lactic acid. In the absence of heat of neutralization of the acid both phases would be endothermic and if the lactic acid is neutralized there would be a negative followed by a positive phase. One discrepancy here is in the disregard for the heat of neutralization of pyruvic acid: it would be justified only if the pyruvic acid had a fleeting existence and this would conflict with the conception of a two stage reaction. An elementary test of the above theory is to find the effect of blocking the cycle at a point prior to the formation of pyruvic acid: this has been done and shows that not only the negative phase but also the early part of the ensuing positive phase are not to be attributed to lactic acid production.

It was shown by Lundsgaard [1934] that the phosphocreatine breakdown (liberating 22,000 cal./g.mol.) occurs partly *after* the contraction. It is coupled with the resynthesis of adenosine triphosphate (absorbing 24,000 cal./g.mol.). The total heat from this coupled reaction is just negative. It is suggested, therefore, that the negative phase immediately following the contraction is caused by this coupled reaction. As the muscle becomes fatigued it is known that the resynthesis of adenosine triphosphate becomes less complete and this may account for the early heat *output* in a fatigued muscle. As further evidence the results of a subsequent paper may be mentioned: it will be shown that with a muscle at pH 6 an early alkalinity change, attributable to breakdown of phosphocreatine, is half complete in about 1 min. which is the time taken for half completion of the "alactacid" anaerobic heat.

In the same paper it will be seen that the formation of lactic acid has also been followed. At 0° C. it is found to be very much slower than was previously suspected, being half complete in about 20 min. The time course of its formation is obviously different from that of the anaerobic heat production considered as a whole, but it seems possible that it may coincide with the slow second phase. The total heat evolved in this second phase is small (about 5% of the initial heat) and spread over more than 30 min.: it is difficult to measure it accurately. As a means of detecting lactic acid production, measurement of the associated heat is practically valueless owing to the endothermic coupled resynthesis of



phosphocreatine which almost exactly brings the total heat output to zero [Lundsgaard, 1931]. It is probable that the anaerobic recovery heat production under normal conditions continues longer than is shown here: it may be that the observer wrongly attributes the whole of the galvanometer deflection at 30–40 min. to osmotic changes in the muscle. Further experiments using paraffin oil in the muscle chamber may show that the recovery heat really proceeds for about 60 min., a result which would be more in harmony with the conclusions of the next paper.

### SUMMARY

1. A study has been made at 0° C. of the anaerobic recovery heat of frog's muscle following a tetanus of 12 sec. duration.

2. A better prospect of interpreting the results is afforded by the demonstration that the early stages of the recovery heat production are unaffected when lactic acid formation is prevented.

3. Under "alactacid" conditions the time course of the recovery heat production follows a regular sequence of variation with successive runs. Following the first tetanus in nitrogen the recovery heat may be entirely negative: after the third there may be an early negative followed by a positive phase and after the ninth there is solely a positive phase. In every case the heat production is complete in 5–10 min.

4. Under normal conditions there is, in addition, a slow phase of heat production attributable to lactic acid formation. This phase has indefinite onset and continues for at least 30 min. The total heat evolved in this second phase is only about 5% of the initial heat and there is doubt about the accuracy with which it has been measured.

5. The probable significance of the early phase of the anaerobic recovery heat is discussed.

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## HYDROGEN-ION CONCENTRATION CHANGES IN FROG'S MUSCLE FOLLOWING ACTIVITY

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THE  $pH$  changes in stimulated muscle have previously been followed by three methods:

- (1) By measuring carbon dioxide gas exchange.
- (2) By the glass electrode.
- (3) By indicators [Margaria & Pulcher, 1934].

The attributes of the first two methods will be discussed briefly.

(1) *Carbon dioxide exchange.* The method is due to Lipmann & Meyerhof [1930]. The muscle was stimulated in fluid at room temperature and carbon dioxide exchange between the muscle and gas space was necessarily slow. The stimulation was in short bursts at regular intervals and the readings were taken at half-hour stages. There was no possibility of investigating the kinetics of the changes following a single short tetanus. It was not possible to study the changes with oxygen present probably for the following reasons: (1) there is complication due to usage of oxygen and production of carbon dioxide; (2) the reactions occur so quickly at room temperature that oxidative recovery would be sufficiently rapid to keep the muscle in a "steady state" with the rate of stimulation employed. No overall changes would be observed and the time lag is too great for transient ones to be measured. With a muscle in oxygen it is possible to stimulate it so much that the total catabolism shall progressively increase; but finally, at room temperature and with a muscle as thick as a frog's gastrocnemius or sartorius the rate of diffusion of oxygen would be insufficient to supply the needs of the innermost layers.

(2) *The glass electrode.* The method employed by Dubuisson [1937, 1939] has the advantages of being specific to  $pH$  changes and of rapidity in recording. The only time lag is caused by transmission of changes across the layer of fluid immediately surrounding the muscle. It is not possible to follow  $pH$  changes during oxidative recovery.

The method to be described here depends upon carbon dioxide exchange but differs essentially from that of Lipmann & Meyerhof [1930] because the muscle is supported in the gas space. This so reduces the time lag as to permit investigations of the kinetics of the changes following a single tetanus and of the changes occurring with oxygen present. Diffusion in the muscle is allowed for by calculation: errors so introduced are rendered unimportant by lowering the temperature to 0° C. The rate of metabolism is thus reduced much more than the rate of diffusion. Events during the contraction are much too transient to be studied by this means. For measuring short period changes (lasting a few seconds) the glass electrode method is clearly superior, but for recording pH changes which accompany some recovery events in frog's muscle at 0° C. the method to be described here is preferable owing to the stability of the base line over periods of 60 min. or more. For some of the slow changes which are investigated there is practically no correction for diffusion.

#### METHOD

Two identical frog's sartorii are fitted into the bulbs of a differential volumeter. The bulbs are filled with a mixture containing carbon dioxide. Either muscle can be stimulated and the resulting gas exchange is registered by observing the movements of the index drop in the capillary. All details of the apparatus and method of recording are given elsewhere [Hill, 1940a]. The volumeter was kept at 0° C. except where otherwise stated.

*Control of pH. Factors governing the rate of exchange of CO<sub>2</sub> following a change in pH.* For the following reasons it is important that the initial pH of the muscles be known. (1) The pH change due to hydrolysis of phosphocreatine is dependent upon the initial pH [Meyerhof & Lohmann, 1928]. (2) Lactic acid production is inhibited by a low pH [Kerly & Ronzoni, 1933].

The solution used for the preliminary soaking is brought to the required pH with bicarbonate-CO<sub>2</sub> buffer. The CO<sub>2</sub> mixture used for bubbling through the solution is the same as is used for filling the chambers of the volumeter. The range of pH required is 5-9. The concentrations of bicarbonate and CO<sub>2</sub> required are calculated from the following equilibrium relations:

$$\frac{[\text{CO}_2]}{[\text{H}_2\text{CO}_3]} = 700 \quad \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} = 2 \times 10^{-4}.$$

The solubility coefficient of CO<sub>2</sub> = 1.

An isotonic solution of sodium bicarbonate is prepared and the calculated quantity added to Ringer's solution. Not more than 40% of the soaking mixture must be isotonic bicarbonate or the muscle develops spontaneous twitching: thus to work at pH 10 it is necessary to use only 0.04%  $\text{CO}_2$ .

At a given pH it is desirable, for the following reasons, to buffer with high concentrations both of bicarbonate and  $\text{CO}_2$ . (1) With a very small percentage of  $\text{CO}_2$  there is a danger of the supply of  $\text{CO}_2$  in the chamber being exhausted. A muscle at pH 6 with oxygen uptake inhibited develops irreversible alkaline changes on stimulation, and about 1 mm.<sup>3</sup>  $\text{CO}_2$  is absorbed following a 6 sec. tetanus. The volume of each bulb of the volumeter is 5 c.c. If five runs are taken the gas mixture must not contain less than about 0.1%  $\text{CO}_2$ . (2) When an equivalent  $X$  of acid is suddenly liberated in the muscle  $fX$  of  $\text{H}_2\text{CO}_3$  is formed immediately. The rate at which  $\text{CO}_2$  is liberated will be proportional initially to  $fX$ . The fraction  $f$  is less than, but approaches, unity as the efficiency of buffering increases. A similar argument applies to an alkaline change. It can be shown that in the range of buffer mixtures that are practically available at pH 6 the value of  $f$  may rise from 0.4 (for 1%  $\text{CO}_2$ ) to 0.93 (for 50%  $\text{CO}_2$ ). In conjunction with this it is important to consider the rate of the reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2$ . The concentration of carbonic anhydrase in frog's muscle is 2-3% of that in blood and the reaction in either direction is therefore 90% complete in a few seconds. Provided the buffering is sufficiently good, there will only be a few seconds' delay in translation of a pH change into an equivalent change in  $\text{CO}_2$  concentration.

## RESULTS

- (1) pH 9 with normal oxygen consumption.
- (2) pH 6-7.5 with normal oxygen consumption.
- (3) pH 6 with oxygen consumption completely inhibited by cyanide.
- (4) pH 6.5-8.5 with oxygen consumption completely inhibited by cyanide.
- (5) pH 6.5-8.5 with oxygen consumption partially inhibited by sodium azide.

### (1) pH 9 with normal oxygen consumption

At pH 9 there is practically no development of alkalinity accompanying breakdown of phosphocreatine. Following a tetanus of 6 sec. there is, however, a slow absorption of gas which is not complete for 20-30 min. (Fig. 1). The explanation is that the respiratory quotient of an active muscle is 0.9 [Gemmill, 1934] and therefore the volume of oxygen absorbed during recovery is greater than the volume of  $\text{CO}_2$  produced. By finding the oxygen consumption under the same conditions it has been shown that the magnitude of the change observed is consistent with this value of the respiratory quotient.

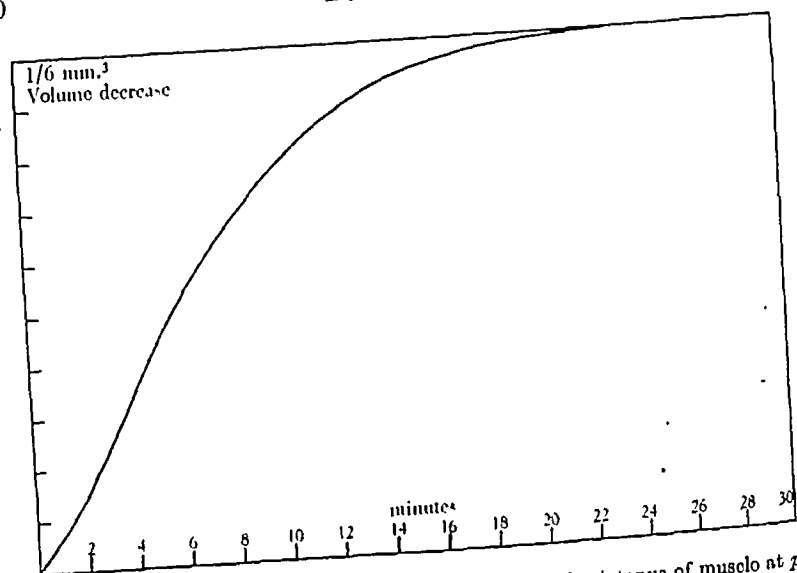


Fig. 1. Record of movement of index drop following 0.2 min. tetanus of muscle at pH 9 in an atmosphere of oxygen and  $\text{CO}_2$ . Oxygen consumption not inhibited. Muscle 80 mg. Total volume decrease  $\frac{1}{6}$  mm.<sup>3</sup>

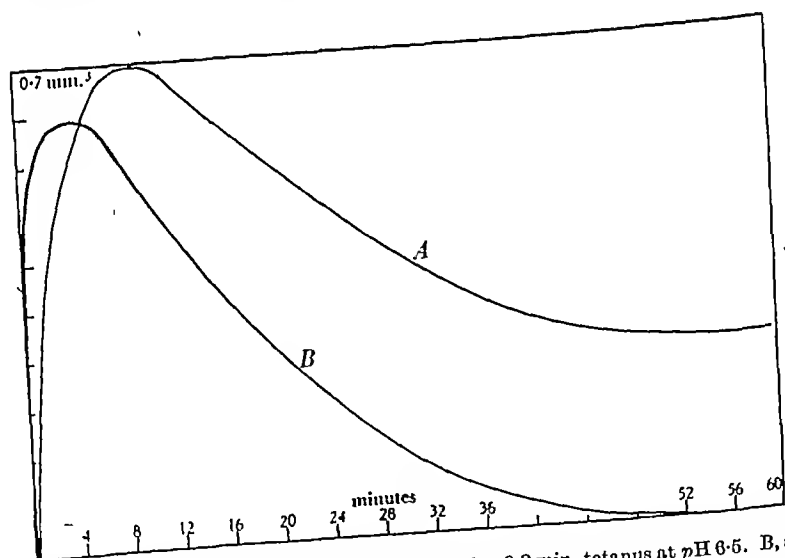


Fig. 2. A, record of movement of index drop following 0.2 min. tetanus at pH 6.5. B, after correction for oxygen consumption and  $\text{CO}_2$  production (by subtraction of curve shown in Fig. 1) and for diffusion. Weight of muscle 100 mg.

## (2) pH 6-7.5 with normal oxygen consumption

The curve followed by the index drop after a 3 sec. tetanus is shown in Fig. 2. The absolute height of the alkaline peak is dependent upon the initial pH since it is partly due to hydrolysis of phosphocreatine. The early rate of absorption is so rapid that the initial heat expansion is completely obscured. Resynthesis of phosphocreatine is slow and the index drop does not finally come to rest for 30-40 min. The final displacement of the base line is determined by the respiratory quotient.

To obtain the time course of change of pH the following corrections must be made: (1) For heat production of the muscle. (2) For diffusion of  $\text{CO}_2$  in the muscle. (3) For the combined effects of utilization of oxygen and production of  $\text{CO}_2$ . The corrections (1) and (2) are made in a manner described in a previous paper [Hill, 1940a] and correction (3) is effected by subtracting the curve shown in Fig. 1 after scaling for equal final maxima. The results of this analysis is also shown in Fig. 2. The curve represents the balance between two processes, the breakdown and resynthesis of phosphocreatine. At the plateau the rates of the two processes are equal. The next experiment gives a better indication of the time course of breakdown since the complication due to resynthesis is avoided.

In the past the aerobic resynthesis of phosphocreatine has been followed by chemical methods, but no exact time course has been obtained. Neither volume change methods nor the glass electrode can be used on a muscle with adequate oxygen supply. Von Muralt [1934] measuring light transparency changes in muscle was able to follow phosphocreatine resynthesis in a muscle fully supplied with oxygen. He found that, at 20° C., the resynthesis following a 2 sec. tetanus is complete in 5 min. With a temperature coefficient of 2.5 for 10° C. this would be in agreement with the results obtained here.

## (3) pH 6 with oxygen consumption completely inhibited

Kerly & Ronzoni [1933] have shown that lactic acid production in minced muscle is inhibited by acidifying the medium to pH 6. It will be seen that this is true also for intact stimulated muscle which has been soaked in a solution buffered at pH 6. Inhibition of lactic acid production by this means is preferable to the use of iodoacetic acid because the objectionable effects associated with the latter's action are avoided. A muscle can be kept at pH 6 indefinitely without damage provided oxygen is present: resynthesis of phosphocreatine occurs normally as shown in (2).

The oxygen uptake is inhibited by soaking for 1 hr. in Ringer's solution containing  $M/1000$  sodium cyanide. Fig. 3 shows the result of a 3 sec. tetanus. It is seen that there is neither production of lactic acid nor resynthesis of phosphocreatine. The analysis in this case simply consists in correction for diffusion and for the initial heat production. The diffusion constant used here and in (2) is derived from the value found by Wright [1934] for frog's muscle at  $22^{\circ}\text{C}$ . by allowing a 1% decrease per  $1^{\circ}\text{C}$ . fall in temperature. In Krogh's units the diffusion

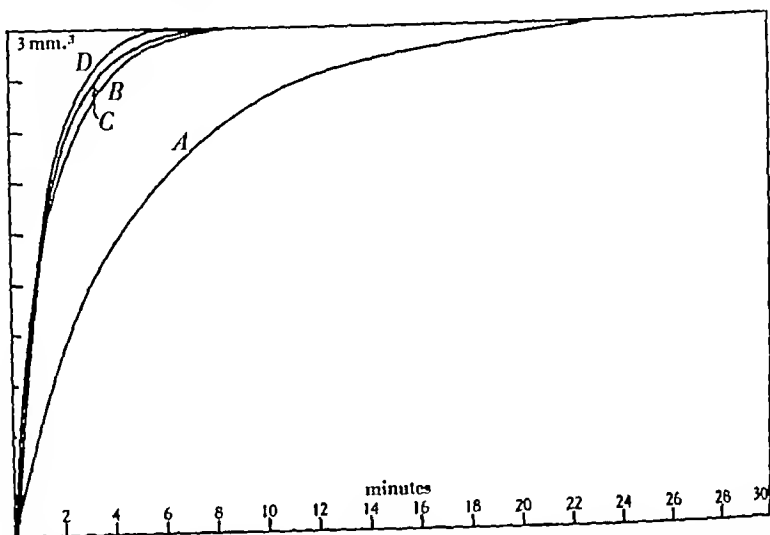


Fig. 3. *A*, absorption of  $\text{CO}_2$  following 0.2 min. tetanus at pH 6. Temperature  $0^{\circ}\text{C}$ . Oxygen uptake inhibited by cyanide. Thickness of muscle 0.9 mm. *D*, absorption of  $\text{CO}_2$  following  $\frac{1}{2}$  sec. tetanus at  $16.3^{\circ}\text{C}$ . *C*, the same but with times multiplied by the ratio of diffusion constants at  $16.3^{\circ}\text{C}$ . and  $0^{\circ}\text{C}$ . *B*, calculated control curve for thickness 0.9 mm.

constant at  $0^{\circ}\text{C}$ . is  $5.3 \times 10^{-4}$ . The calculated curve for instantaneous disappearance of  $\text{CO}_2$  is also shown in Fig. 3. It has not been found possible to obtain this control curve experimentally. The nearest approach that can be made is by stimulating the muscle under the same conditions but at a much higher temperature. The curve resulting from a  $\frac{1}{2}$  sec. tetanus at  $16.3^{\circ}\text{C}$ . is also shown in Fig. 3. It was with the same muscle as had been used at  $0^{\circ}\text{C}$ . For comparison with the calculated curve the abscissae are multiplied by the ratio of the diffusion constants at  $16.3$  and  $0^{\circ}\text{C}$ . The result shows that there will be no gross error from using the calculated curve for the analysis.

The curve corrected for diffusion is shown in Fig. 4. There appears to be a *sudden* alkaline change during activity followed by a slower change which is not complete for more than 10 min. It is probable that the curve gives the time course of phosphocreatine breakdown. Lundsgaard [1934] has shown that a certain fraction of phosphocreatine breakdown occurs *after* the relaxation, and the alkalinity phase which Dubuisson [1939] attributes to the same cause occurs *after* the relaxation.

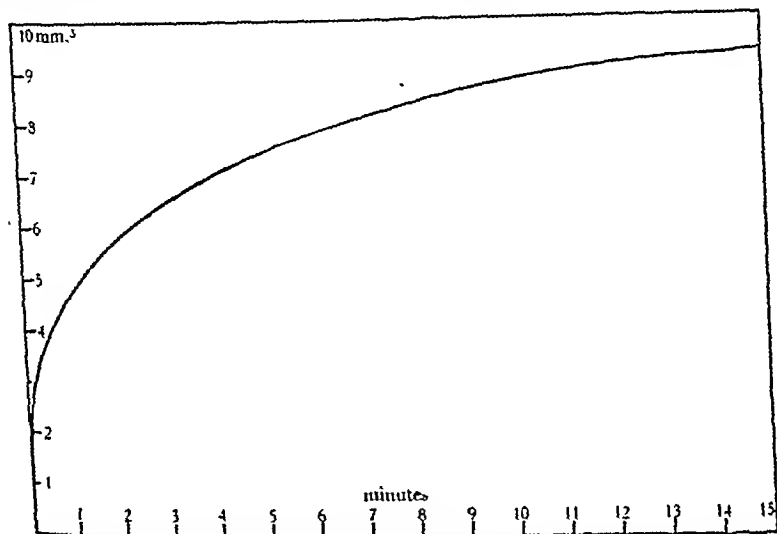


Fig. 4. Time course of the alkaline change following 0.2 min. tetanus. The conditions are such that oxygen consumption and lactic acid production are inhibited.

There is a marked resemblance between the analysed curve of Fig. 4 and the curve of anaerobic delayed heat production for a late member of a series under "alactacid" conditions [Hill, 1940*b*]. Both curves are half complete in about 1 min. It was suggested that the early stages of the anaerobic delayed heat accompanied the breakdown of phosphocreatine.

(4) pH 6.5–8.5 with oxygen consumption completely inhibited by cyanide

In this range of pH lactic acid production is not inhibited. To lessen, but not to abolish, the effect due to breakdown of phosphocreatine a pH of 7 is chosen and the effect of stimulation is shown in Figs. 5 and 6. There is first a heat expansion (at this pH it is not obscured by the concomitant alkaline change): next an absorption of  $\text{CO}_2$  which starts rapidly but soon slows up and then reverses. The curve recrosses the



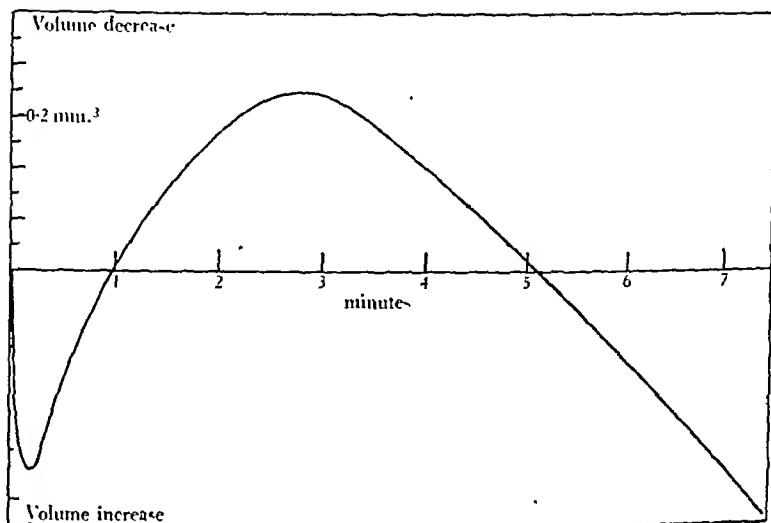


Fig. 5. Early stages of  $\text{CO}_2$  exchange following 0.2 min. tetanus of muscle at pH 7 with oxygen consumption completely inhibited by cyanide.

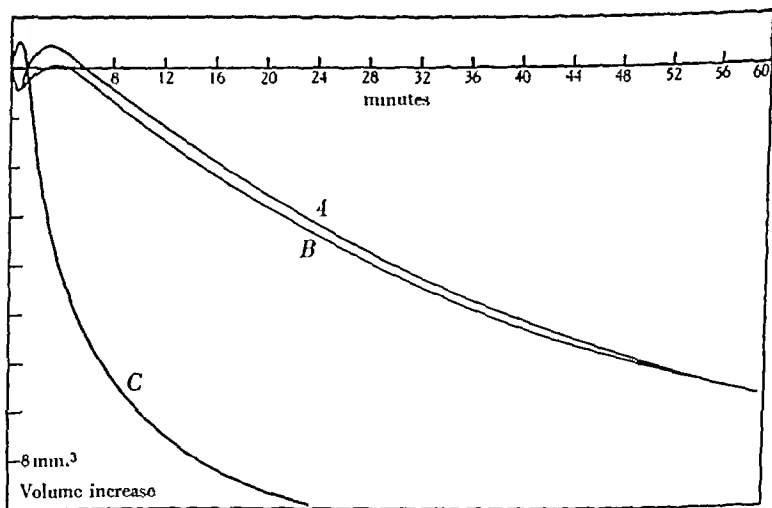


Fig. 6.  $\text{CO}_2$  exchange following tetanus of muscle with oxygen uptake completely inhibited by cyanide. *A*, complete record at pH 7; the early stages are shown on a larger scale in Fig. 5. *B*, complete record at pH 8.5. *A* and *B* are both at  $0^\circ \text{C}$ . with 0.2 min. tetanus. *C*, at pH 7.5,  $17^\circ \text{C}$ . following a 1 sec. tetanus.

base line at about 5 min. and the output of  $\text{CO}_2$  then continues for more than 1 hr. At pH 8.5 there is no change of pH due to breakdown of phosphocreatine: the initial heat expansion in that case is followed by a return toward the base line as the heat is dissipated, but the acidity reversal supervenes before the base line is crossed.

There can be little doubt that the prolonged acidity expansion is due to lactic acid formation. It is impossible to say exactly where this phase commences owing to the complication, in the early stages, due to the heat expansion and phosphocreatine breakdown. There would be no advantage in correcting the curve for diffusion, since only the early part where rapid changes are occurring would be materially affected and it is this region which has doubtful significance owing to summation of coincident changes.

*The same experiment at 17° C.* The form of the curve is the same as at 0° C. but with the time axis diminished (Fig. 6). For the same total lactic acid production the rate of formation at any time is about 5 times greater at the higher temperature.

The time course of lactic acid production has not previously been studied at 0° C. At room temperature the subject has been tackled by various methods: (1) chemical analysis [Lehnartz, 1931; Meyerhof, 1931a, b, c; Lundsgaard, 1931]; (2) volume change [Meyerhof, 1934]; (3) light transmission [Von Mural, 1934]; (4) glass electrode [Dubuisson, 1937, 1939; Dubuisson & Schulz, 1938]. None of these methods, however, has been capable of determining the time course accurately. Dubuisson, for example, has stated nothing more explicit than that the acidity change is complete "within a few seconds" after contraction.

(5) pH 6.5-8.5 with oxygen consumption partially inhibited by azide

Activity oxygen consumption can be inhibited to any extent desired by poisoning with the appropriate concentration of sodium azide [Hill, 1940a]. It was therefore anticipated that progressive application of azide to a muscle at pH 7 should result in a continuous change from the aerobic type of curve (Fig. 2) to the anaerobic type of curve (Fig. 6). This proved not to be the case. The results using 3 sec. tetani are shown in Fig. 7. The azide concentration was increased from  $M/50,000$  to  $M/4000$  (with  $M/4000$  the activity oxygen consumption is practically abolished). With  $M/50,000$  azide the aerobic type of curve was obtained showing a slow resynthesis of phosphocreatine. As the concentration of azide was increased the only change observed was a decrease in the rate of resynthesis. Even with  $M/4000$  azide the lactic acid phase was

completely absent. The addition of cyanide restored the ability to form lactic acid.

Recovery oxidation can, therefore, be completely stopped and yet no lactic acid is formed when the muscle is stimulated for 3 sec. A possible explanation might be that resting oxygen consumption (which is not abolished by azide [Stannard, 1939] is sufficient to destroy lactic acid or its precursors as fast as they are formed. If this is the case an increase in the duration of stimulus should raise the rate of production

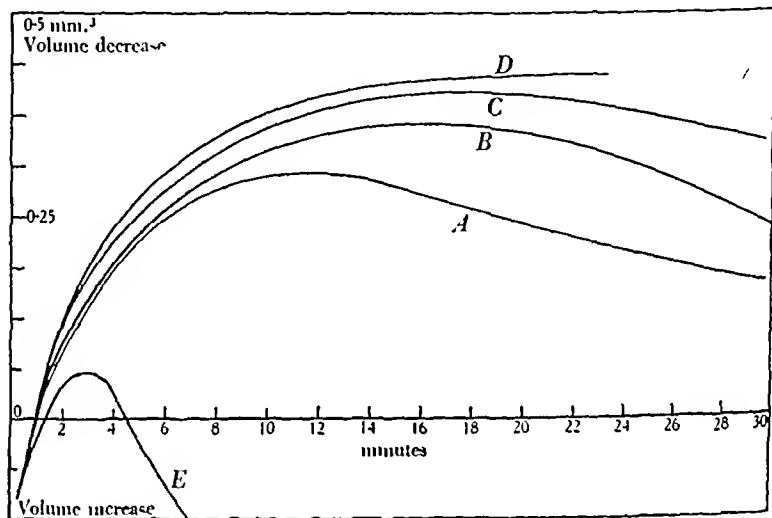


Fig. 7. Carbon dioxide exchange of muscles with oxygen consumption partly inhibited by azide. pH 7. 0.05 min. tetanus. A,  $M/50,000$  azide; B,  $M/30,000$  azide; C,  $M/15,000$  azide; D,  $M/4000$  azide. Compare E, oxygen consumption completely inhibited by  $M/1000$  cyanide. Even with  $M/4000$  azide there is no lactic acid production following a 0.05 min. tetanus.

of lactic acid (or its precursors) to a level at which the small resting oxygen consumption is incapable of maintaining a steady state. Lactic acid would then have to accumulate. A muscle, treated with  $M/4000$  azide was stimulated for 3, 12 and 36 sec. with intermediate soaking at room temperature to hasten the later stages of recovery. The early stages of the resulting curves are shown in Fig. 8. After a 3 sec. tetanus there is no progressive lactic acid formation: with a 12 sec. tetanus the lactic acid phase appears, the curve crossing the base line at about 10 min.: with a 36 sec. tetanus the curve crosses the base line at 4 min. and is now similar to that obtained when oxygen consumption is completely

abolished by cyanide. In the last case the effect of the residual resting oxidation is negligible, the rate of production of lactic acid having been raised overwhelmingly.

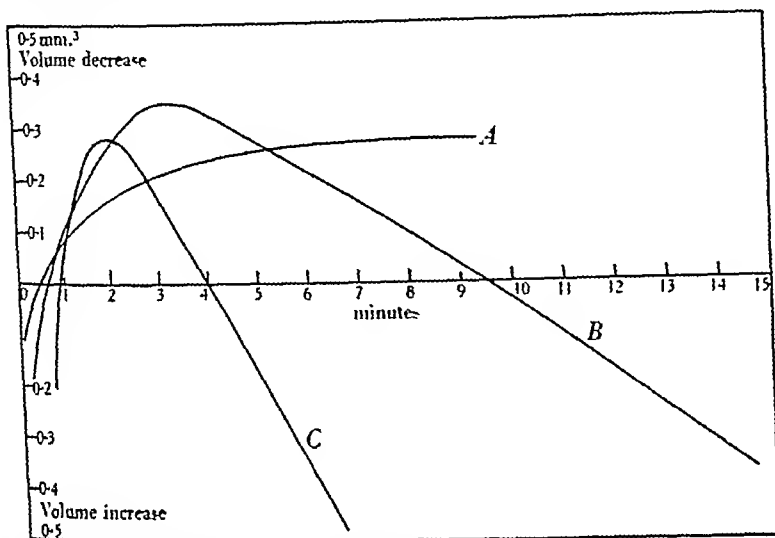


Fig. 8. Effect of changing duration of tetanus with muscle poisoned by  $M/4000$  azide. A, 3 sec. tetanus; B, 12 sec. tetanus; C, 36 sec. tetanus.  $0^{\circ}\text{C}$ ., pH 7.

### DISCUSSION

It has long been known that, in a muscle fully supplied with oxygen, there is no lactic acid formation following a short tetanus. This fact was frequently used as evidence for the theory that lactic acid is the substrate normally oxidized in a stimulated aerobic muscle. Other theories now hold sway but it is interesting to observe that the results of this paper militate against the older view. The argument is as follows. It has been seen in a previous paper [Hill, 1940*a*] that the rate of oxygen consumption following a short tetanus is at a maximum near the beginning of recovery and declines approximately exponentially. In other words, the activated substrate which is oxidized is formed rapidly during, or closely after, the contraction. On the other hand, lactic acid is formed extremely slowly. There is no possibility, therefore, that lactic acid is identical with the substrate normally oxidized in aerobic activity.

The conclusion must be that the formation of lactic acid occurs along a branch reaction which is independent of the main channel of oxidation. This is in harmony with present views on the chemistry of muscle.

It should not be inferred that lactic acid or its precursors cannot be oxidized. On the contrary, there is no reason to suppose that the reactions tending to the formation of lactic acid are not pressing forward even when oxidative recovery is permitted and the non-appearance of lactic acid must be due to oxidation of lactic acid or its precursors at the rate of formation. This subsidiary oxidation must be partly via cytochrome (for inhibition of cytochrome greatly facilitates the production of lactic acid) and partly via another respiratory enzyme refractory to the poisoning action of sodium azide. This latter system is capable of destroying lactic acid (or its precursors) at the rate at which it is formed following a 3 sec. tetanus at 0° C. but cannot restrain the accumulation of lactic acid after a longer tetanus. Comparison with the results of a previous paper [Hill, 1940a] shows that anaerobic recovery with lactic acid formation is considerably slower than aerobic recovery under the same conditions. The oxidation system concerned with prevention of lactic acid formation is thus presumably of *minor importance*. It should be considered as a mechanism for disposing of the end-products from non-oxidative recovery reactions. When oxygen is present these recovery processes are assisted by the more rapid ones which are primarily dependent on the progress of an oxidative reaction.

#### SUMMARY

1. Changes in *pH* of stimulated frog's muscle are followed by the method of carbon dioxide exchange using a differential volumeter.
2. The muscle is supported in the gas space and it is possible to make the necessary corrections for diffusion and so obtain the time course of *pH* changes following a single short tetanus. Greater accuracy in this respect is attained by working at 0° C. and so slowing the metabolic processes. Changes of *pH* during the tetanus cannot be followed by this method.
3. When oxygen usage is permitted or in other circumstances when lactic acid is not formed (e.g. anaerobically at *pH* 6) alkaline changes only are observed. These accompany the breakdown and resynthesis of phosphocreatine and the time courses of these processes can be deduced.
4. At *pH* 7 with oxygen uptake inhibited by cyanide the production of lactic acid is not complete for more than 1 hr. It is half complete in about 15 min.
5. When the activity oxygen uptake is inhibited by sodium azide (the resting oxygen uptake being unaffected) the time course of formation

of lactic acid is dependent upon the duration of tetanus. If the latter is reduced below a critical value no lactic acid is formed. The significance of this is discussed.

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## SLOW VAGINAL POTENTIALS

By R. B. BOURDILLON AND O. M. LIDWELL

*From the National Institute for Medical Research, Hampstead, N.W. 3**(Received 6 June 1940)*

A NUMBER of workers have described slow potential changes occurring simultaneously with the contractions of unstriated muscle [Alvarez, 1922], [Alvarez & Mahoney, 1922], [Berkson, Baldes & Alvarez, 1932], [Funke, 1921]. Such potentials are especially liable to include artefacts due to movement at or near the junction between tissues and electrodes. Bozler [1939] has recently concluded that the slow potentials observed by himself when using thread electrodes for intestinal contractions were artefacts of this type. It seems of importance to establish the existence of these slow potentials under conditions as far as possible free from artefacts, and we have found vaginal potentials on rabbits especially suitable for such studies since they can be examined without any surgical interference or exposure of tissues normally covered by skin. We have therefore made tests of this question by taking serial radiographs of vaginal contractions simultaneously with potential measurements, using electrodes in a number of different sites.

## METHOD

Adult rabbits were used in various stages of the pregnancy cycle, with dial anaesthesia. The methods were similar to those described previously [Bourdillon, 1939], except that a cathode ray oscillograph was used instead of a galvanometer. The electrode wires led to the paraphase input stage, Toennies type [Matthews, 1938], of a four-stage direct coupled amplifier. One electrode was earthed, all (low tension) wires to the X-ray plant were wrapped with earthed aluminium foil, and the amplifier enclosed in earthed steel boxes. With these precautions the 50 cycles interference during the X-ray exposures was reduced to 1/10 mV. as seen in Fig. 5, or sometimes even less.

*Radiography.* The radiographs were taken with a Victor portable 60 kV. 10 mA. unit on Ilford X-ray paper, which was used in rolls of 100 ft.  $\times$  4 $\frac{1}{2}$  in. An anterior intensifying screen was used in a camera constructed so that the manual movement of a lever released a pressure plate, moved on the paper, and reapplied pressure to the paper at a rate permitting exposures of  $\frac{1}{4}$ -1 sec. to be made at intervals of about 3 sec. We are greatly indebted to Dr Schuster for the design and construction of this camera and of the one used for recording potentials. In all tests the vagina was partly or wholly filled with about 15 c.c. of saline (NaCl 9 g., KCl 0.42 g., dist. H<sub>2</sub>O 1 l.) containing barium sulphate and

gum arabic. This was introduced by a catheter round which the outermost part of the vaginal canal was tied so as to prevent extrusion of fluid during contractions.

*Electrodes.* In series A the electrodes consisted of a no. 10 catheter in the vagina and a glass tube of 4-6 mm. bore with an open end held in contact with the skin. In series B and C both electrodes were glass tubes held on the skin. The catheter or glass tubes were full of saline and led to AgCl-Ag electrodes. In series D wire electrodes were used as shown in Fig. 7.

## RESULTS

*Series A.* One vaginal electrode (catheter) and one glass tube electrode on the skin of the thigh, or substernally.

Pl. I, fig. 1 shows a radiograph (no. 10) taken at the moment of maximum negativity of the vaginal potential curve shown in Fig. 2. The catheter electrode can be seen extending from the right-hand side into the vagina for about 8 cm. The catheter orifice appears as a dark area immediately to the right of a solid plug of barium sulphate (white) which had been cemented into the tip of the catheter. Directly above the catheter orifice is a dark shadow between two balloon-shaped portions of the vagina to the right and left. This dark shadow represents a contraction wave just passing the catheter orifice from left to right. Above the vagina various parts of the uterus are visible owing to barium which passed through the uterine aperture. Fig. 3 shows outline-drawings of the radiographs taken during this potential wave. The exact time and duration of exposure of each radiograph is shown on the potential curve (Fig. 2) by horizontal black lines at the top and bottom of the record. No. 8 (vaginal potential normal) shows the vagina well dilated opposite the catheter orifice, although a contraction wave has reached the catheter tip. In no. 9 the potential is one-third of the way down, and the contraction wave is just reaching the catheter orifice. In no. 10 (the radiograph shown in Fig. 1) the contraction wave is just passing the catheter and the potential is at maximum negativity. In nos. 11-13 the potential is rising and the contraction is moving farther beyond the orifice. No. 14 shows almost maximal positivity of potential and maximal relaxation of the vagina as shown by the bulge below the catheter orifice.

The close synchronism shown between contractions and potentials is very different from the irregular results which would be expected from movement artefacts, or even from streaming potentials caused by the passage of liquid past the catheter orifice. It does not however exclude the possibility that the potentials arise in the vaginal epithelium rather than in the muscle, and are influenced or even caused by friction of the epithelium on the catheter. Accordingly further tests were made as follows without any electrode in the vagina.



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*Series B.* One substernal electrode and one on the abdominal skin about  $\frac{1}{2}$  in. proximal to the symphysis pubis.

Pl. II, fig. 4 shows a radiograph (no. 58) taken at the moment of maximum negativity of the first potential wave shown in fig. 5. The lower end of the glass tube which acts as the abdominal electrode is seen at *E*. A contraction wave is seen below and to the right of this electrode. Fig. 6 shows outline drawings of a series of radiographs taken during the two potential waves shown in Fig. 5. In series of this type using an extra-abdominal electrode we have often found that the moment of maximum negativity is slightly before or after the moment of passage of the contraction wave under the electrode. Any non-uniform resistance of tissues could cause this, and we believe it is due to the intervention of a pad of fat which is commonly found ventral to the inner end of the vagina in pregnant rabbits, and extends for varying distances towards the symphysis. This fat can evidently cause the electrical path of least resistance between a fixed point on the abdominal skin and the vagina to be inclined at an angle to the perpendicular. In Fig. 6 the apparent line of least resistance is shown by the arrows. These are drawn at the same angle in each sketch. If the angle differed erratically between the different radiographs of any one series of potential waves, such variation would provide a powerful argument against the correlation of potential waves with contractions. In our experiment, however, these angles have been constant for any one rabbit, unless the active electrode was moved.

Results of the above type have been obtained in numerous rabbits, and show that the slow negative potentials associated with the contraction are not dependent on the use of an internal electrode. Visual observation and tests made by manual movement of the abdomen show that the potentials are not due to obvious movement of the skin at the abdominal electrode. It might however be argued that the pressure under this rigidly held electrode must vary considerably during the passage of a vaginal contraction wave, and that the observed potentials might be due to such pressure changes. To test this a trial was made as follows:

*Series C.* One substernal electrode, and one over the hamstring and adductor muscles of thigh.

The left thigh was firmly held between the electrode and one side of the tray supporting the rabbit. Hence no movement of the thigh was visible even in the more vigorous contractions where appreciable movement of the abdomen was seen.



Fig. 1.

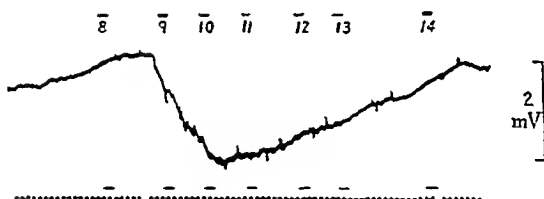
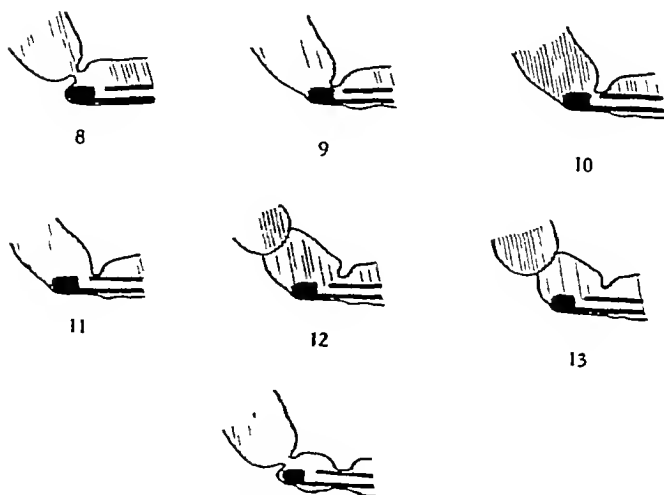


Fig. 2.



corded from a metallic electrode in contact with a moving muscle must be open to grave suspicion of being influenced by artefacts. It is possible that Fig. 8 is so influenced, but we are inclined to think that the major

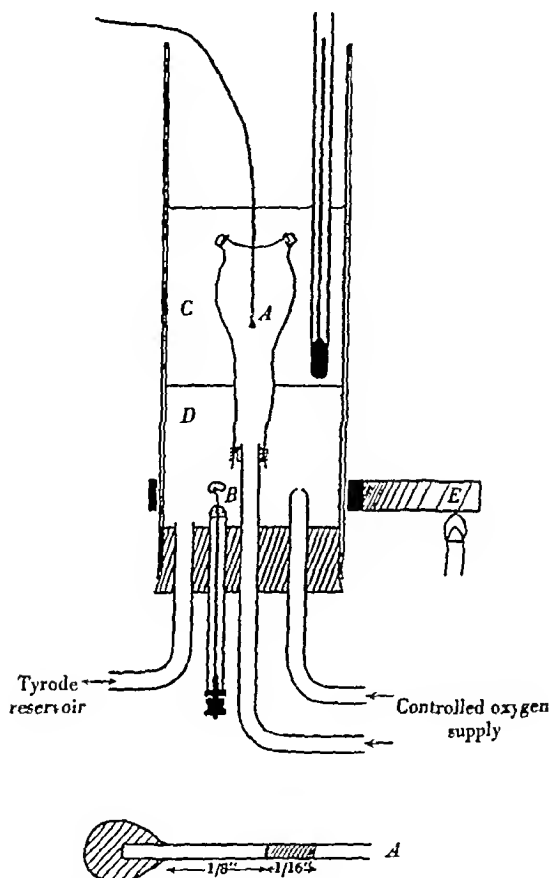


Fig. 7. Arrangement of apparatus for recording potentials from isolated vagina. *A*, micro-electrode of 42 s.w.g. enamelled copper wire; end immersed in a ball of insulating cement; insulation removed for  $1/16$  in. from section near end and wire silvered. Inserted just within the serous coat of the vaginal wall parallel with the longitudinal fibres. *B*, electrode of chlorided silver wire, *A* and *B* connected to input side of amplifier. *C*, layer of medicinal paraffin floating on tyrode solution. *D*, *E*, copper band heated by gas flame maintaining temperature near  $38^{\circ}\text{C}$ .

features of the two waves shown do represent genuine changes in the potential of the contracting portion of the vaginal muscle (or changes in the ionic content of the muscle electrolyte).

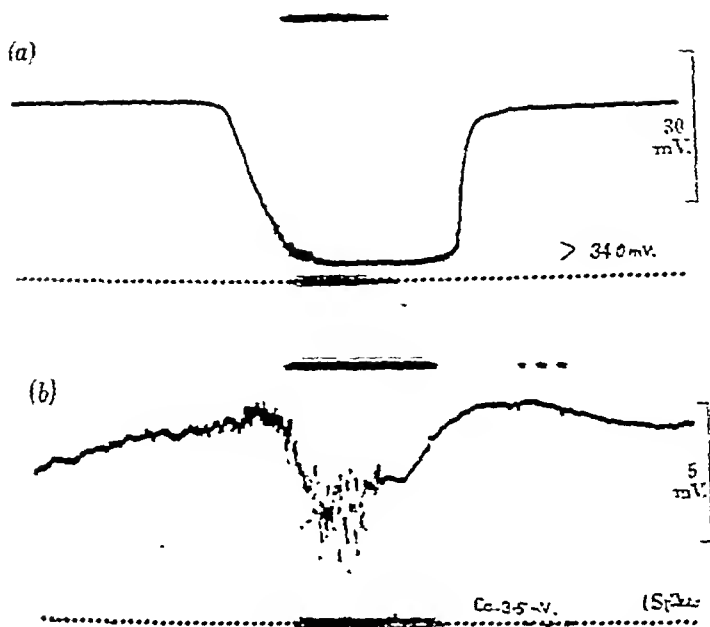


Fig. 5. Potential waves recorded from isolated vagina. Downward movement of the line indicates increased negativity of the vaginal electrode. The long horizontal black lines at the top and bottom of the record indicate the period of maximal visible contraction at the electrode. Time marks at  $\frac{1}{2}$  sec. intervals.

### DISCUSSION.

In series A, B and C we have obtained some 182 radiographs showing good correspondence with seventy-four potential waves in a number of different rabbits. When considered together we think the above experiments give strong support to the view that the slow potential changes recorded are not artefacts. The chief artefacts that tend to arise in such experiments with saline electrode leads have been avoided as follows:

(1) Movement of partly dried peritoneum or other internal tissue surface. In tests of type A, B and C no surgical interference has occurred (except a stitch round the labia in A and B), and no internal surfaces are exposed.

(2) Movement of skin in contact with the electrode. This could only have occurred in series B and C. Careful observation showed that such movement was not visible in the vast majority of the tests and that small movements made by hand tended to cause potential changes smaller than those recorded during contractions.

(3) Changes of pressure under the electrode. Such changes must have occurred in series A and B, but cannot have occurred to any appreciable extent in C when leading off the thigh.

The results in series D from the isolated vagina have not been fully confirmed, but are shown since the large values recorded under paraffin are such as might be expected from unstriated muscle with the contracted portion in a state of partial insulation from its surroundings.

### SUMMARY.

Simultaneous radiographs and potential records have been taken showing contractions of the vagina in rabbits. Close synchronism of contraction and potential fall have been obtained with the active electrode in the vagina, or on the skin of the abdomen, or on the thigh.

Some potential curves are shown from a silver electrode in an isolated vagina contracting in paraffin. These show a maximum negativity exceeding 34 mV.

The above data are considered as tending to confirm the theory that contraction waves in unstriated muscle are accompanied by negative potential waves lasting for a number of seconds.

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## EXPLANATION OF PLATES I AND II

### PLATE I

- Fig. 1. Radiograph of vagina and catheter electrode at the moment indicated by the number 10 in Figs. 2 and 3. Barium in the vagina appears grey.  
 Fig. 2. Potential wave between vagina and skin of rabbit. In this and Fig. 5 the horizontal black lines at the top and bottom of the record show the occurrence and duration of X-ray exposures (the numbers referring to the corresponding drawings in Figs. 3 and 6). Time marks are at  $\frac{1}{2}$  sec. intervals, and downward movement of the line indicates increased negativity of the vagina.  
 Fig. 3. Outline drawings of radiographs taken during the potential wave shown in Fig. 2. In this and Fig. 6 the cross shading indicates the barium sulphate in saline which filled the vagina.

### PLATE II

- Fig. 4. Radiograph of vagina at moment indicated by the no. 58 in Figs. 5 and 6.  
 Fig. 5. Potential waves between substernal and abdominal skin of rabbit.  
 Fig. 6. Outline drawings of radiographs taken during the potential waves shown in Fig. 5.

THE ABSORPTION OF WATER FROM THE COLON  
OF THE RAT UNDER URETHANE ANAESTHESIA

BY B. L. ANDREW, J. N. DAVIDSON AND R. C. GARRY

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(Received 20 June 1940)

THE colon of the rat under urethane anaesthesia can absorb only negligible amounts of monosaccharides [Davidson & Garry, 1939]. In view of the rather unexpected nature of this result, we decided to investigate the powers of the colon to absorb other substances, in particular water. Moreover, we wished to make sure that the colonic loops in such rats had still an active circulation and that absorption was therefore possible.

Three methods of attack were chosen. In the first place, since sugars are not absorbed from the colon, the variation of concentration of sugar in a closed loop of colon will give an indication of the movement of water into or out of the loop. Secondly, a non-absorbable indicator will reveal increase or decrease in the concentration of the contents of the loop in similar fashion. The indicator used was phenol red first recommended by Goreham in 1923, and subsequently used by a large number of investigators, e.g. Wilhelmj, Neigus & Hill [1933], Penner, Hollander & Saltzman [1938], as a dilution indicator in gastric analysis. The concentration of phenol red employed was too low to have any appreciable effect on the osmotic pressure of the solution. Thirdly, deuterium oxide may be used as an index of water exchange as has been done in the case of the small intestine by Hevesy & Hofer [1934], McDougall, Verzár, Erlenmeyer & Gaertner [1934] and by Peters & Visscher [1939].

Distilled water alone cannot be used since it damages the mucous membrane [Dennis, 1940]. We therefore introduced as solute either glucose or xylose in all experiments. No attempt was made to measure the actual volume of solution in the colon at the end of 90 min., since we found it difficult to express all the fluid from the loop.



## METHODS

Rats were given water but no food for 48 hr. and were then anaesthetized with urethane given subcutaneously in a dose of 1.6 mg./g. body weight. One hour later the abdomen was opened by a mid-line incision and the lower end of the ileum was tied off. An opening was made into the tip of the caecum and into the colon at its junction with the caecum. Colon and caecum were then washed out with Ringer's solution at 38° C. Thereafter a purse-string suture was inserted round the anus and tightened on a small glass cannula. A second cannula was inserted into the proximal end of the colon and all traces of Ringer's solution washed out of the colon with a stream of fluid containing hyper-, iso- or hypotonic sugar solution at 38° C. In the majority of experiments this sugar solution contained phenol red. The anal cannula was then withdrawn and the purse-string suture tightened. The colon was allowed to fill with solution, the upper cannula was withdrawn and the opening ligatured. In many cases the caecum was similarly washed out and filled with sugar solution. The abdomen was closed and the animal left in warm surroundings for 90 min. At the end of that time the rat was killed, the colonic loop and the caecum were removed, opened and the contents drained out. Reducing sugar was estimated by the method of Hagedorn and Jensen and phenol red by the colorimetric method of Hollander, Penner & Saltzman [1937].

In the experiments with deuterium oxide the colon alone was used. After the colon had been washed out with Ringer the bulk of the residual liquid was removed with a few puffs of air. 5 ml. of a 4% solution of xylose in water containing approximately 20% D<sub>2</sub>O were then run repeatedly through the colon. The anal suture was tightened and about 3 ml. of the solution left in the colon which was then tied off as before. The remaining 2 ml. were preserved for control estimations of sugar and D<sub>2</sub>O.

At the end of a given time the animal was killed, the colonic loop removed, and the contents drained out. For the deuterium oxide estimations portions of about 1 ml. of the fluid under investigation were distilled *in vacuo* at 20° C. into a receiver cooled in a freezing mixture. When all the water had passed over, the distillate was allowed to come to room temperature and the D<sub>2</sub>O content was measured in a Pulfrich refractometer. Control experiments were carried out to ensure that no interfering substances were carried over in the distillation of fluid from colonic loops.

# RESULTS

In all cases where the original solution was hypertonic (10.0%) the contents of the colon at the end of 90 min. were much less concentrated. Since passage of sugar out of the colon does not occur there must have been passage of water into the loop. When phenol red was present as well as glucose, the extent to which this indicator was diluted corresponded closely to the degree of dilution of the glucose (Table I).

The opposite effect was seen where the initial glucose solution was hypotonic (2.7%), the solution undergoing concentration during the 90 min. in the colon. The concentration of phenol red rose correspondingly (Table I).

TABLE I. Variations in the concentration of the contents of colonic loops in rats under urethane anaesthesia in the course of 90 min. The figures given are average values obtained from several experiments.

Glucose concentration g./100 ml.			Phenol red concentration mg./100 ml.		
Initial	Final		Initial	Final	
$c_1$	$c_2$	$c_2/c_1$	$c_1'$	$c_2'$	$c_2'/c_1'$
10.0	6.5	0.65	5.0	3.5	0.70
5.4	4.8	0.89	5.0	4.3	0.86
2.7	3.8	1.41	5.0	7.1	1.42

Where the initial glucose concentration was 5.4%, the value usually considered to be isotonic with mammalian blood [Verzár & McDougall, 1936], the concentration within the colon after 90 min. was slightly lower, about 4.9%. This was not due to loss of sugar since the phenol red concentration fell to a similar degree (Table I).

Similar results were obtained with the caecum.

In the experiments with heavy water, xylose was used as the non-absorbable sugar. Xylose behaved in exactly the same way as glucose, and the strength of solution neither concentrated nor diluted was found to be 4.0%. This, as in the case of glucose, is lower than the concentration (4.5%) said to be isotonic with blood.

We therefore used a 4.0% solution of xylose in water containing about 20.0% D<sub>2</sub>O. The diminution in concentration of D<sub>2</sub>O due to exchange of deuterium atoms with the hydrogen atoms of the hydroxyl groups of xylose was negligible. Moreover any rapid exchange of deuterium atoms with hydrogen atoms present in the colon mucous membrane was allowed for by preliminary repeated passage of the solution through the loop. Although measurements of heavy water concentration by a refractometer are not particularly delicate, yet the

concentration changes we observed were so gross that the method was perfectly adequate for the purpose.

The xylose concentration, originally in the neighbourhood of 4.0%, remained at that level throughout the absorption period which varied from 5 to 90 min. We can therefore assume that the total amount of water in the colon remained constant. Deuterium oxide disappeared very rapidly from the colon; in 30 min. the bulk had gone, and even in as short a time as 5 min. a large proportion had disappeared (Fig. 1).

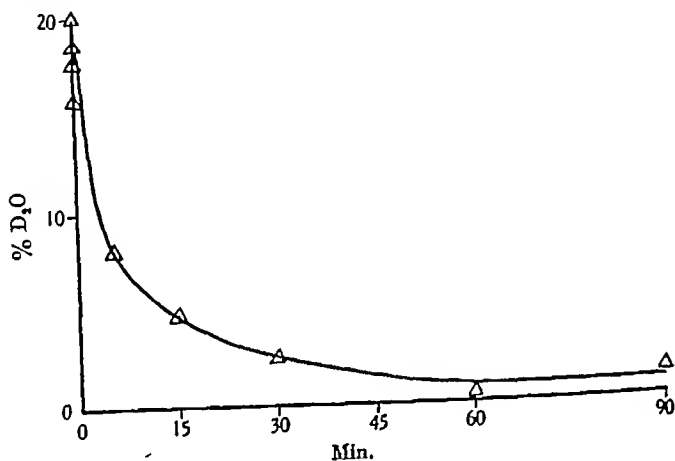


Fig. 1. Decrease in the concentration of heavy water in the contents of colonic loops in the anaesthetized rat.

### DISCUSSION

There can be no doubt that the colonic mucous membrane in rats under the conditions of our experiments allows mass movement of water in both directions while remaining impermeable to monosaccharides. This latter property is lost when the colon is isolated *in vitro* [Davidson & Garry, 1939]. Even in the absence of an osmotic gradient, exchange of water molecules between the blood and the colonic contents was very rapid. The colonic mucous membrane seems to act as an extremely efficient semi-permeable membrane and there can no longer be any suspicion that the failure to absorb monosaccharides was due to experimental conditions interfering with the state of the colon.

# SUMMARY

The colon of the rat under urethane anaesthesia, although impermeable to monosaccharides, is freely permeable in both directions to water molecules.

We wish to express our thanks to Prof. W. F. K. Wynne-Jones for supplying the deuterium oxide and to the Carnegie Trust for the Universities of Scotland for an expenses grant for this work.

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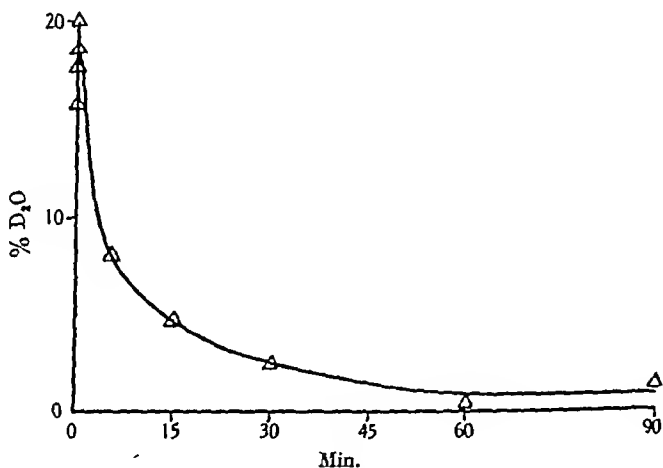


Fig. 1. Decrease in the concentration of heavy water in the contents of colonic loops in an anaesthetized rat.

### DISCUSSION

There can be no doubt that the colonic mucous membrane in vivo under the conditions of our experiments allows mass movement of water in both directions while remaining impermeable to monosaccharides. This latter property is lost when the colon is isolated *in vitro* [Davidson & Garry, 1939]. Even in the absence of an osmotic gradient, exchange of water molecules between the blood and the colonic contents was very rapid. The colonic mucous membrane seems to act as an extremely efficient semi-permeable membrane and there can no longer be any suspicion that the failure to absorb monosaccharides was due to experimental conditions interfering with the state of the colon.

It appears, therefore, that there must exist in the guinea-pig uterus some mechanism for the production of an adrenaline contraction, and that there must also be some unappreciated factor accounting for the irregularity of the results obtained. The following observations are concerned with a hitherto undescribed modification of the action of adrenaline upon the guinea-pig uterus and a possible factor in adrenaline reversal is discussed.

### METHODS

The apparatus used was similar to that described by Pittenger [1928]. A guinea-pig was killed by a blow on the head and exsanguinated. One horn of the isolated uterus was suspended in a bath of oxygenated Ringer-Locke solution contained in a water-bath which was kept at a temperature of  $37.5^{\circ}\text{C}$ . within limits of  $\pm 0.1^{\circ}$ . The uterine movements were recorded by means of an isotonic lever, the tension exerted on the uterus by the recording system being about 1 g. In some experiments both horns of the uterus were used, the bath of Ringer-Locke solution and the recording lever being duplicated to give two identical but independent preparations. The composition of the Ringer-Locke solution was as follows:  $\text{NaCl}$  0.9 g.,  $\text{KCl}$  0.042 g.,  $\text{CaCl}_2$  0.024 g.,  $\text{MgCl}_2$  0.00025 g.,  $\text{NaHCO}_3$  0.05 g., dextrose 0.05 g., distilled water to 100 c.c. All the chemicals used were of Analytical Reagent standard of purity, and the water was double-distilled, the second time in an all-glass still. The adrenaline used was the 0.1% solution of adrenaline chloride (Parke Davis) in appropriate dilution. Any possible effect of the chlorotone present in this solution as a preservative was excluded by control experiments.

### RESULTS

Most of the experiments were made upon the uteri of sexually immature guinea-pigs of from 180 to 250 g. in weight. A uterine horn from such an animal, when placed in the bath of Ringer-Locke solution and attached to the recording lever, relaxed almost completely within a few minutes and showed only small to very small spontaneous contractions. Adrenaline added to the bath to give a concentration of between 1 in 1 million and 1 in 2 millions caused immediate complete relaxation of the uterus and inhibition of spontaneous movement; this lasted for 10 or 15 min. after which a slow recovery began. Upon removal of the adrenaline solution from the bath and its replacement by fresh Ringer-Locke, the uterus very rapidly regained its former degree of tonus and movement.

## THE ACTION OF ADRENALINE UPON THE UTERUS OF THE GUINEA-PIG AND ITS MODIFICATION BY ESERINE

By W. T. AGAR<sup>1</sup>

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*(Received 26 June 1940)*

THE effect of adrenaline upon the isolated uterus of the guinea-pig is considered by most authors [Sugimoto, 1913; Gunn & Gunn, 1914; and many others subsequently] to be inhibition and relaxation whether the animal is pregnant or not. Another view which has been put forward is that the action of adrenaline on the guinea-pig uterus is reversed, that is, changed from inhibition to contraction, during oestrus [Kochmann & Seel, 1929], oestrus and late pregnancy [Heller & Holtz, 1932], oestrus, early and late pregnancy, but not mid-pregnancy [Holtz & Wollpert, 1937]. Such results are not in agreement with those of the majority of investigators, and, indeed, the tracings published by these authors do not present clear-cut evidence of uterine contractions in response to adrenaline.

A number of authors have described a reversal of the action of adrenaline upon the guinea-pig uterus after subjecting the uterus to various procedures of a nature designed to increase the irritability of the uterine muscle. Cow [1919] and McSwiney & Brown [1926] produced adrenaline reversal by treating the uterus with posterior pituitary extract, but this effect could not be obtained by Athias [1921] or Holtz and Wollpert [1937]. McSwiney & Brown also produced reversal by leaving the uterus for 1 hr. in cold Ringer-Locke solution. Many authors have kept uteri at room temperature or in the ice-chest for much longer periods without noticing this change (for instance, Gunn & Gunn [1914]). Tate & Clarke [1922] found adrenaline reversal after exposure of the guinea-pig uterus to ergot; this result has not been confirmed.

<sup>1</sup> Nuffield Dominion Demonstrator.

After the uterus had been exposed to the action of eserine for from  $1\frac{1}{2}$  to 3 hr. it was observed, in fifteen out of seventeen experiments in which sexually immature guinea-pigs were used, that a change had appeared in the reaction to adrenaline. That concentration of adrenaline which had previously produced prolonged inhibition and relaxation now produced inhibition and relaxation followed after a varying period by a contraction. At its first appearance the contraction was small and followed a period of inhibition lasting 6 or 8 min. As the exposure of the uterus to eserine was prolonged, the change in the reaction to adrenaline became progressively more marked until it reached a maximum. At this stage a suitable concentration of adrenaline (usually between 1 in 1 million and 1 in 2 millions) caused relaxation and inhibition of the uterus for 3-5 min. followed by a maximal contraction which subsided spontaneously after 5 or more min. (Figs. 1, 2). Occasionally, especially in the more muscular uteri, the secondary contraction persisted for 30 min. or more. Within limits, the size of the contraction varied with the concentration of adrenaline, a smaller concentration causing a smaller contraction, though the duration of the initial period of relaxation did not vary appreciably. The initial phase of relaxation was made more striking if a small degree of tonus was produced in the uterus by a suitable dose of pituitary extract or acetylcholine before the adrenaline was added to the bath (Fig. 3). In some experiments both horns of the uterus were used, two identical preparations being set up in different baths, and one horn was treated with eserine while the other was left untreated. After the lapse of from  $1\frac{1}{2}$  to 3 hr. the eserinated horn responded to adrenaline by relaxation followed by contraction, the untreated horn always responded by simple relaxation.

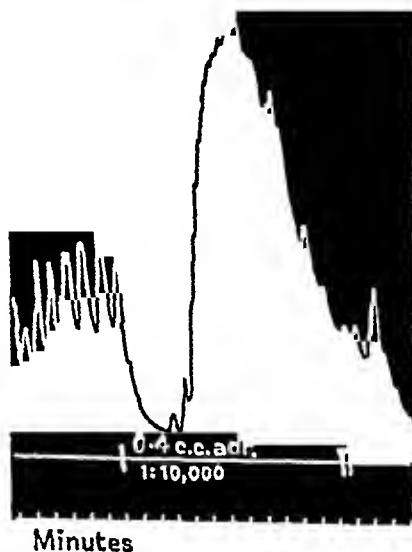


Fig. 3. Diphasic effect of adrenaline, 1 in 1.8 millions. The uterus had been exposed to eserine, 1 in 2.8 millions, for 120 min. The initial tonus was produced by adding to the bath acetylcholine, 1 in 350 millions, a few minutes before the adrenaline. Signals as before.



The solution surrounding the uterus was then changed for Ringer-Locke solution to which had been added eserine salicylate in a concentration of 1 part in  $1\frac{1}{2}$ -2 millions. Exposure of the uterus to such a solution, prolonged often up to 4 or 5 hr., did not appear to have any toxic effects. The spontaneous movements of the uterus were either not



Fig. 1.

Fig. 1. Diphasic effect of adrenaline, 1 in 1.8 millions, after exposure of uterus to eserine, 1 in 2.8 millions, for 150 min. Single signal, adrenaline added to bath; double signal, fresh Ringer-Locke.

Fig. 2. Diphasic effect of adrenaline, 1 in 1.3 millions, after exposure of uterus to eserine, 1 in 1.6 millions, for 160 min. Signals as before.

tered or else showed a slight increase in amplitude. The response to pituitary (posterior lobe) extract (Burroughs Wellcome) was not changed, qualitatively or quantitatively. The sensitivity of the uterus to acetylcholine was greatly increased, after 20 or 30 min. exposure to eserine the uterus usually responded with a large contraction to a concentration of acetylcholine of 1 part in 200 millions. In the untreated uterus a concentration of from 10 to 40 times this figure was necessary to produce a comparable contraction.

once occurred, and upon again changing the solution a second "lavage" contraction appeared.

When a uterus was showing the diphasic response to adrenaline, removal of the adrenaline solution during the initial phase of relaxation had variable results. Sometimes no contraction followed, sometimes a "lavage" contraction immediately appeared. In general, the later the adrenaline was removed during the phase of relaxation the larger was the resulting contraction, when present. That is, removal of the adrenaline often precipitated a contraction which would have occurred later in the presence of the adrenaline.

*Experiments with "Substance 36".* The "Substance 36" of Aeschlimann & Reinert [1931] is the phenylmethyl carbanic ester of 3-hydroxy-phenyl-trimethyl-ammonium methyl sulphate. It closely resembles prostigmine in chemical constitution; both substances, like eserine, contain the urethane grouping which is believed to be responsible for the inhibition of choline esterase [Stedman & Stedman, 1932]. In three experiments both horns of the uterus were set up to form identical preparations, one horn was treated with eserine, 1 part in  $1\frac{1}{2}$  millions, and the other with Substance 36 in the same concentration. In all three experiments a diphasic reaction to adrenaline appeared in both horns of the uterus without significant difference in the length of exposure necessary to produce the reaction or in the nature of the reaction when it appeared. The potentiating effect of Substance 36 upon the response to acetylcholine was the same as that of the same concentration of eserine.

*Effect of atropine.* Atropine hydrochloride in a concentration of 1 part in 120,000 did not alter within 30 min. the diphasic response of the uterus to adrenaline, whether this response had been evoked by treating the uterus with eserine or with Substance 36. This concentration of atropine abolished within 8 min. the response of the eserinated uterus to a concentration of acetylcholine which had previously caused a large contraction.

*Effect of ergotoxin.* The effect of ergotoxin on the diphasic response of the eserinated uterus to adrenaline was not easy to determine, as exposure of the uterus to ergotoxin frequently caused a persistent, nearly maximal, increase in tonus. In two favourable experiments, in which exposure of the eserinated uterus to ergotoxin ethane sulphonate in a concentration of 1 part in 3 millions, for 8 and 24 min. respectively, caused no more than a moderate persistent tonus, it was possible to show that the diphasic response of the uterus to adrenaline was not altered. Exposure of the

A variant of the diphasic response of the eserized uterus to adrenaline, which was seen in two experiments, was the appearance of a contraction only when the adrenaline was removed from contact with the uterus. In such a case adrenaline produced only relaxation and inhibition, but immediately the solution in the bath was changed a large contraction appeared (Fig. 4). Changing the solution never caused a contraction in the absence of an antecedent dose of adrenaline. This

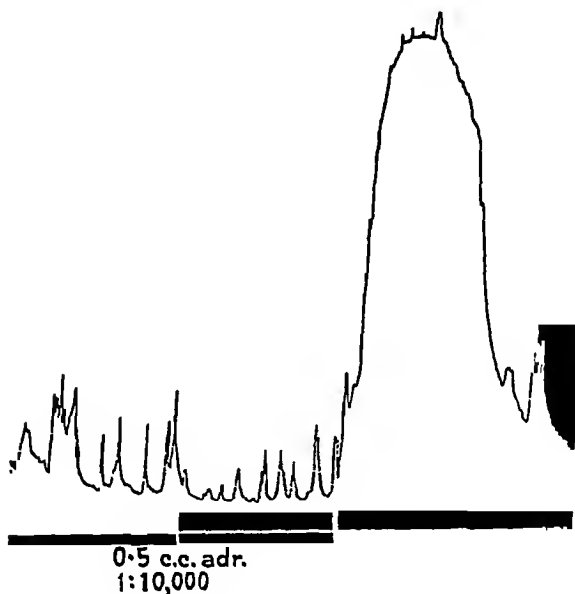


Fig. 4. "Lavage" contraction on removal of adrenaline, 1 in 1.4 millions, from contact with the uterus. Eserine, 1 in 2.8 millions, for 105 min. Signals as before.

"lavage" contraction after adrenaline was exactly the same as that described by Launoy [1933] as an occasional finding in the guinea-pig uterus, especially after exposure to pituitary extract.

If a second dose of adrenaline was added to the bath during the phase of contraction produced by a previous dose of the same size, relaxation immediately occurred, though it was not always complete. After 3 or 4 min. this was followed by a second contraction, that is, the second dose of adrenaline caused the repetition of the diphasic phenomenon. This was also the case if a second dose of adrenaline was added to the bath during a "lavage" contraction following a previous equal dose. Relaxation at

and two in late pregnancy. Oestrus was recognized by the vaginal smear, in two experiments it was induced by injection of oestrogens, in the other it was spontaneous.

All these uteri showed considerably greater spontaneous activity than the infantile uterus. The dioestrous uteri showed small to moderate rhythmic contractions, the uteri of oestrus and early pregnancy frequent irregular maximal contractions, and the uteri of late pregnancy occasional maximal contractions separated by long periods of quiescence. In each case the response to adrenaline was relaxation and inhibition, though the adult uteri, whatever their physiological state, were considerably less sensitive to adrenaline than the infantile uterus. A concentration of 1 part in 2 millions caused prolonged inhibition and relaxation of the latter, from 4 to 8 times this concentration was necessary to produce a comparable effect upon the former. The uteri of late pregnancy were even less sensitive to adrenaline. A concentration of 1 part in 200,000 had no discernible effect during a quiescent period of the uterus; after a series of rhythmic contractions had been provoked by a small concentration of pituitary extract this concentration of adrenaline caused only a very slight decrease in amplitude and increase in frequency of the contractions.

Prolonged eserization produced a diphasic response to adrenaline in two out of the six dioestrous uteri. In the oestrous and pregnant uteri eserine did not alter the response to adrenaline.

### DISCUSSION

It appears from the foregoing results that adrenaline has a twofold action on the eserinated uterus of the sexually immature guinea-pig in that it causes an immediate inhibition followed by a delayed contraction. The initial inhibitory phase shows no discontinuity with the simple inhibition obtained in the untreated uterus, and in producing this effect adrenaline is no doubt acting upon the receptive substance of muscle cells with an inhibitory sympathetic innervation [Langley, 1905].

The fact that the secondary contraction appears under the influence both of eserine and of a prostigmine-like substance which share the property of inhibiting choline-esterase, but are otherwise very different in chemical constitution, suggests that it is this property which is responsible for the altered reaction of the uterus to adrenaline. This invites the hypothesis that the secondary adrenaline contraction is due to a release of acetylcholine. This hypothesis is consistent with the less constant appearance of a secondary adrenaline contraction in conditions

uterus of the rabbit to this concentration of ergotoxin abolished within 5 min. the motor response of that organ to a concentration of adrenaline equal to that used in the experiments on the eserinizd guinea-pig uterus.

*Effect of nicotine.* Nicotine in a concentration of 1 part in 80,000 did not alter the diphasic response of the eserinizd uterus to adrenaline.

*Effect of potassium.* The concentration of potassium chloride in the Ringer-Locke solution was 420 mg./l. Doubling this concentration led to a small increase in the frequency and amplitude of the spontaneous movements of the uterus and a slight increase in tonus; trebling it caused a moderate to submaximal contraction. On restoring the original Ringer-Locke the uterus rapidly regained its former state.

In three out of five experiments in which the uterus was exposed to a doubled or trebled concentration of potassium for 20 or 30 min. the reaction to adrenaline was seen to be diphasic after restoration of the original Ringer-Locke. The diphasic effect appearing after exposure to potassium was less well marked than that appearing after eserine, in that the secondary contraction was smaller and less abrupt. Exposure of the uterus to excess of potassium also markedly increased its sensitivity to acetylcholine, though not to the same extent as did eserinization.

The sensitivity of the uterus to potassium was found to increase spontaneously by about 50% on repeated testing in four experiments; in one experiment no increase occurred. In four experiments prolonged exposure of the uterus to that concentration of eserine which produced a diphasic response to adrenaline did not cause any further increase in the sensitivity to potassium.

*Other causes of a diphasic reaction to adrenaline.* It was occasionally observed that an untreated uterus which had been suspended in the bath for a long time showed a small secondary contraction after the initial inhibition produced by adrenaline. The uterus in such a case showed signs of irritability in increased spontaneous activity and increased sensitivity to acetylcholine.

Cocaine hydrochloride in a concentration of 1 part in 20,000 increased the spontaneous activity and tonus of the uterus and slightly increased the sensitivity to acetylcholine. In one experiment out of three a diphasic response to adrenaline appeared. Cocaine had no influence upon the diphasic response to adrenaline appearing after eserinization.

*Experiments on the adult uterus.* The effect of eserine upon the response to adrenaline of the dioestrous, oestrous, and pregnant uterus of the guinea-pig was studied. Six experiments were made during dioestrus in animals of over 350 g. weight, three during oestrus, one in early pregnancy,

and two in late pregnancy. Oestrus was recognized by the vaginal smear, in two experiments it was induced by injection of oestrogens, in the other it was spontaneous.

All these uteri showed considerably greater spontaneous activity than the infantile uterus. The dioestrous uteri showed small to moderate rhythmic contractions, the uteri of oestrus and early pregnancy frequent irregular maximal contractions, and the uteri of late pregnancy occasional maximal contractions separated by long periods of quiescence. In each case the response to adrenaline was relaxation and inhibition, though the adult uteri, whatever their physiological state, were considerably less sensitive to adrenaline than the infantile uterus. A concentration of 1 part in 2 millions caused prolonged inhibition and relaxation of the latter, from 4 to 8 times this concentration was necessary to produce a comparable effect upon the former. The uteri of late pregnancy were even less sensitive to adrenaline. A concentration of 1 part in 200,000 had no discernible effect during a quiescent period of the uterus; after a series of rhythmic contractions had been provoked by a small concentration of pituitary extract this concentration of adrenaline caused only a very slight decrease in amplitude and increase in frequency of the contractions.

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### DISCUSSION

It appears from the foregoing results that adrenaline has a twofold action on the eserinated uterus of the sexually immature guinea-pig in that it causes an immediate inhibition followed by a delayed contraction. The initial inhibitory phase shows no discontinuity with the simple inhibition obtained in the untreated uterus, and in producing this effect adrenaline is no doubt acting upon the receptive substance of muscle cells with an inhibitory sympathetic innervation [Langley, 1905].

The fact that the secondary contraction appears under the influence both of eserine and of a prostigmine-like substance which share the property of inhibiting choline-esterase, but are otherwise very different in chemical constitution, suggests that it is this property which is responsible for the altered reaction of the uterus to adrenaline. This invites the hypothesis that the secondary adrenaline contraction is due to a release of acetylcholine. This hypothesis is consistent with the less constant appearance of a secondary adrenaline contraction in conditions

which lead to irritability of the uterine muscle and consequent greater sensitivity to acetylcholine. It is noteworthy that the choline-esterase inhibiting drugs have a specific effect in producing the secondary adrenaline contraction and an increased sensitivity to acetylcholine without increasing the general irritability of the uterine muscle. More prolonged eserization is necessary to produce the diphasic reaction to adrenaline than is necessary to cause maximum potentiation of the effect of acetylcholine externally applied to the uterus.

Atropine leaves unaltered the secondary adrenaline contraction while abolishing the effect of externally applied acetylcholine. This does not necessarily exclude the possibility that the secondary adrenaline contraction is due to a liberation of acetylcholine in close propinquity to the receptive substance of the reacting cells [Dale & Gaddum, 1930]. There is, indeed, evidence that in the bitch atropine does not diminish the contraction produced by stimulating cholinergic nerves to the uterus *in vivo* though it abolishes the effect of injected acetylcholine [Sherif, 1935].

The potassium ion is a possible intermediary between adrenaline and acetylcholine. Inhibition of smooth muscle by adrenaline is accompanied by increased polarization of the inhibited cells, involving an influx of potassium ions [Bacq & Monnier, 1935]. Also, potassium salts cause the liberation of acetylcholine in various organs containing cholinergic post-ganglionic nerve endings [Feldberg & Guimaraes, 1936]. Thus it may be postulated that the influx of potassium ions accompanying adrenaline inhibition is capable of liberating a minute amount of acetylcholine in the neighbourhood of the receptive substance of the muscle cells. This amount of acetylcholine is capable of causing a contraction when its destruction by choline-esterase is minimized by prolonged eserization, or, occasionally, when the sensitivity to acetylcholine has increased as part of a general increased irritability of the muscle cell.

If this hypothesis is correct, it might be expected that an increase in the potassium content of the Ringer-Locke solution surrounding the uterus could also lead to the liberation of a small quantity of acetylcholine, and that there would thus be a small acetylcholine component in the contraction caused by potassium. If this were the case the potassium contraction should be augmented by prolonged eserization of the uterus. No evidence of any such augmentation was obtained. It might well be assumed, however, that the rate of increase in the concentration of the potassium ion in close relation to the cell is greater as a result of the increased polarization accompanying adrenaline inhibition than is

the rate of increase that can be obtained by adding potassium salts to the Ringer-Locke solution.

The reaction of the oestrous and pregnant uterine of the guinea-pig to adrenaline has always been found to be inhibition. Eserine has no effect upon this response. It seems to be impossible to produce the diphasic response to adrenaline in uteri showing much spontaneous activity; this may be connected with the decreased sensitivity to the inhibiting action of adrenaline which these uteri show in comparison to the infantile uterus.

In the experiments here reported it has invariably been found that adrenaline in adequate concentration caused an immediate inhibition of the isolated guinea-pig uterus, though under certain conditions a delayed contraction followed. The term "adrenaline reversal" cannot strictly be applied to this modification of the action of adrenaline. The authors cited in the introduction have described adrenaline reversals in which the contraction was not preceded by inhibition. If these reversals were of the same nature as the diphasic modification described above, it seems probable that they were not specific to the various procedures employed but were an indirect consequence of an increased irritability of the uterine muscle. The inconstancy of the results of different authors may well be due to the presence of unrecognized irritating factors in the physiological salines used. Pittenger [1928] has pointed out how sensitive the isolated uterus is to minute amounts of impurities in chemicals or distilled water; sodium chloride is particularly apt to be at fault. The findings of those earlier workers who believed that the isolated guinea-pig uterus was caused to contract by adrenaline may have been due to the use of imperfect physiological salines which irritated the uterus.

#### SUMMARY

1. The inhibition and relaxation which adrenaline causes in the isolated uterus of the infantile guinea-pig was modified, on prolonged treatment of the uterus with eserine, to a diphasic response in which inhibition and relaxation was followed by contraction. The same response to adrenaline was obtained after treatment of the uterus with a prostigmine-like substance.

2. A number of procedures which increased the irritability of the uterine muscle, and its sensitivity to acetylcholine, inconstantly led to the appearance of a diphasic response to adrenaline.

3. The diphasic modification of the action of adrenaline was not altered by atropine or ergotoxin.



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# PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

10 February 1940

**The influence of secretin on gastric secretion.** By C. L. G. PRATT.  
*From the Department of Physiology, University of Oxford*

Secretin is the specific secretagogue for the pancreas; it is well known also to exert a relatively small effect on the secretion of bile. Similarly, bile salts in the circulation excite the liver to secrete bile, and simultaneously induce a slight production of pancreatic juice. Both secretory stimulants provoke, in addition, a very small flow of gastric juice in the fasting cat. The gastric juice produced by bile salt stimulation resembles "histamine juice" in that it contains little or no enzyme. "Secretin juice", on the other hand, is very rich in pepsin.

The effect of secretin on the output of pepsin has been investigated in a series of experiments on fasting cats under chloralose anaesthesia. Under such conditions the stomach generally does not exhibit spontaneous secretory activity. The subcutaneous injection of 1 mg. histamine produces a flow of gastric juice, at first increasing, and then diminishing in velocity, lasting about 4 hr. If the stomach is washed out beforehand, and the pylorus suitably occluded, samples of gastric juice which do not at any time contain a detectable quantity of pepsin may be collected throughout this period through a cannula tied into the stomach.

Pepsin in considerable quantities appears in the juice leaving the stomach within 5 min. of the intravenous injection of 0.1 mg. of a secretin preparation of such strength that 0.1 mg. induces a flow of 2 c.c. of pancreatic juice in 15 min. A concentration of pepsin may be reached comparable to that found after the subcutaneous administration of 5 mg. acetylcholine, after eserizing the animal, a flow of juice having been first established with histamine.

The secretin effect is probably due to direct action on the gastric glands, for the following reasons:

(1) The effect can be maintained indefinitely by repeated administration of secretin.

(2) It is not abolished by atropine.

(3) The smallest dose which has been observed to activate the pancreas likewise causes an output of pepsin.

(4) The effect is not due to some associated substance in the secretin preparation, because it is equally well observed using Ågren's purified preparation, which is now commercially available for clinical use.

(5) An output of pepsin is also observed when a solution of bile salts is placed in the duodenum, thus provoking the absorption of the cat's own secretin.

**Physical fitness of pre-adolescent boys of three socio-economic levels.** (A preliminary study.) By D. BURNS and J. SECKER. *From the Medical School, Newcastle-upon-Tyne, University of Durham*

Three groups of six boys each between the ages of 12 and 14 were selected as the fittest of their type by physical training experts from

A. a Grammar School;

B. a Suburban Secondary School;

C. a Boy Scout Group from an artisan district.

Average of physical characters

Group	Ht. (cm.)	Wt. (kg.)	V.C. (l.)	Si/S	Bc/Ba
A	156	48.3	3.33	44	70
B	157	43.7	3.0	44	70
C	144	34.2	2.65	45	63

Average functional characters

Group	Total strength kg. (Martin)	B.H. sec.	Max. exp. force mm. Hg	F.W. index
A	43	50.6	59.6	71
B	36.5	46.1	53	68
C	20	41.4	39	43.5

Si/S: ratio sitting height to total height.

Bc/Ba: ratio bicristal to biacromial width.

B.H.: breath holding.

F.W. index (Flack-Woolham):

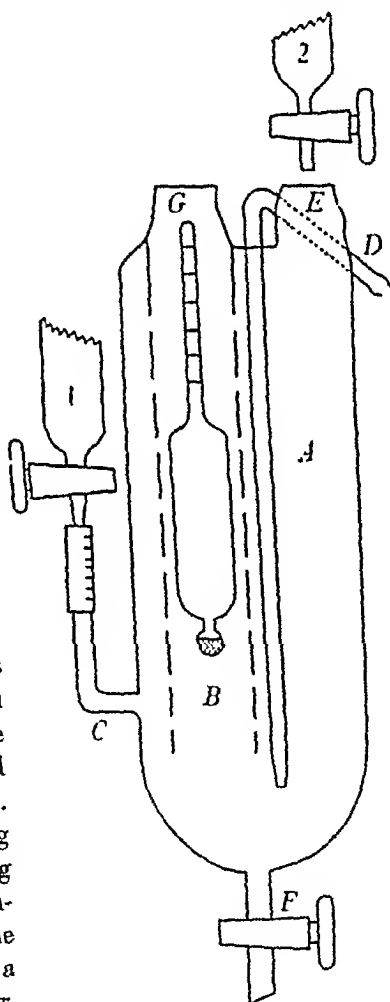
Max. exp. force  $\times$  breath holding (sec.)  $\times$  B.H. (sec.) at half max. exp. force corrected for age by division by  $100 \times (0.25 \text{ age})^{1.807}$ .

Total strength: arbitrary figure (in kg.) from pull of pectorals, forearm flexors and thigh adductors.

**A simple apparatus for the determination of the specific gravity of small amounts of fluid.** By H. HELLER, *Beit Memorial Research Fellow. From the Department of Pharmacology, Oxford*

The apparatus was designed to estimate the specific gravity of biological fluids in cases where the amounts of sample fluid at the disposal of the investigator are less than 0.5 c.c., e.g. catheter urine. Actually 0.1 c.c. of fluid proved to be sufficient. The apparatus obviates the use of a pycnometer in cases where extreme accuracy is not required. It works on the principle that a drop floats in a fluid which has the same sp.gr. and with which it is immiscible.

The ordinary clinical urinometer is used which is kept in tube B. Tube A is filled with a mixture of a heavy and a light fluid, e.g. carbon tetrachloride and toluol or petrol ether. The sp.gr. of this mixture can be decreased by adding the lighter fluid through the capillary tube C, which is connected to burette no. 1. The fluid mixes the content while rising to the surface. The heavier fluid is dropped into tube A from burette no. 2. Facility for a more thorough mixing of the fluids is provided by blowing air through tube D which is connected with a rubber bulb. The sample fluid is dropped from a capillary tube into E, and the sp.gr. of the carbon tetrachloride/toluol mixture varied until a drop remains suspended in the surrounding fluid. (Tube B prevents drops of sample fluid from clinging to the urinometer. It is perforated to permit free mixing of the carbon tetrachloride/toluol mixture.) The urinometer is



then read and the temperature of the mixture taken. Some of the lighter fluid is added if the apparatus is no longer required so that the sample fluid collects above tap *F*. This has the advantage that any used test fluid can be removed when a new determination is begun and that the carbon tetrachloride/toluol mixture can be used for several determinations. When not in use *E* and *G* and the burettes are closed with stoppers to prevent evaporation.

The advantages of the apparatus are:

- (1) Cheapness.
- (2) Use of an ordinary clinical urinometer.
- (3) The small amount of sample fluid needed (0.1 c.c.).

**The potentiation of prostigmine by ephedrine in a case of myasthenia gravis.** By J. W. THORNTON. *From the Pharmacology Laboratory, Bristol University*

Schafer [1939], reporting a case of myasthenia gravis, stated that ephedrine and glycine did not help at all, while prostigmine and benzedrine did so very considerably.

The case here reported is of a girl of 13½ years and 6 stone weight who developed weakness and exhaustion about Easter 1939 when her school teacher noticed drooping eyelids. Benzedrine and glycine were not tried, but the addition of ephedrine to the routine treatment with prostigmine is shown by ergographic records, as well as clinical observation, to increase the power to do muscular work very much.

Ephedrine by mouth had been tried from 20 to 22 October in doses of gr. ¾ t.d.s., and was abandoned because it gave no obvious clinical improvement. When first seen by me on 26 October she was being given guanidine in divided doses of 200 mg. per day by mouth. This had started on 11 October and was stopped on 30 October in order to get control records under prostigmine alone. At this time she was having prostigmine 1 mg. by injection in six 3-hourly doses during the daytime with an increasing number of emergency doses of 0.5 mg. due to threatening respiratory failure as the prostigmine effect was wearing off in less than 3 hr.

Ergographic records were taken from the right middle finger, and it was found that 2½ hr. after prostigmine she could not lift 1 kg. at all. She lifted ½ kg. for 60–70 sec., after which the finger muscles were completely exhausted. Computation of the work done on three such occasions

gave figures of: 25.6; 33.0; 33.2 kg. cm. At this stage an injection of prostigmine 1 mg. became imperative to avoid respiratory failure; 10 min. later she was able to lift the  $\frac{1}{2}$  kg. double the height to begin with and to continue for many minutes without exhaustion or even marked fatigue—in one test she did 72 kg. cm. work in the first minute and then continued for several minutes at a rate of 22.4 kg. cm./min.

When ephedrine was tried in dose of gr. i (65 mg.) by injection instead of the prostigmine the ergogram showed that exhaustion was reached after 80 sec., during which 42.75 kg. cm. work was done. At this point 1 mg. prostigmine was injected and the work done increased in the following way: 85.3 kg. cm. work was done in the 1st minute and the patient continued at a rate of 62.0 kg. cm./min. for 25 min. without any signs of further flagging—and volunteered that she was not tired. Total work done was 1318 kg. cm. This result was obtained on the first occasion when ephedrine and prostigmine were used together, and therefore this combination was continued 3-hourly. By the next day, after five doses of ephedrine and 2-2 $\frac{1}{2}$  hr. after the double injection, the ergogram showed that she could do 608.9 kg. cm. work at an average rate of 35 kg. cm./min. with only partial fatigue. This compares with the 25-38 kg. cm. work she could do at the same stage on prostigmine alone. The next double injection raised the rate of work to 73 kg. cm./min. for 9 min. without fatigue. Two days later there was further improvement, including general condition, and heavier weights could be lifted. 1 kg. was lifted for 11 min. at a rate of 56.7 kg. cm./min. without fatigue, and 2 kg. were lifted for 7 min. at a rate of 122.5 kg. cm./min. when fatigue was almost complete. At this point the total work done was more than 60 times that in controls.

To verify this effect the ephedrine was stopped for 4 days, exhaustion returned and increased prostigmine was necessary. The work done after 1 $\frac{1}{2}$  mg. prostigmine (88 in 1st, 41 in 2nd, 32 in 3rd, and 16 kg. cm. in 4th and 5th min.) was barely more than that in the original controls with 1 mg. prostigmine.

Ephedrine was then restarted in half the previous dosage, i.e. gr. i, 6-hourly (= 3 doses per 24 hr.), leaving the prostigmine as before (1 mg., 3-hourly = 6 doses per 24 hr.). This was continued for 47 days, during which the patient continued to improve clinically and was allowed up at her own discretion, instead of being bedridden. Without warning she collapsed on 8 January, about 6 a.m. and in spite of an extra injection of prostigmine was dead by 6.15 a.m., probably before the injection could take effect.



Analysis of over forty ergograms taken during the course of this case show that the effect of the ephedrine lasted for about 5 hr., and that it did not prolong the action of prostigmine which was still necessary at 3-hourly intervals in the daytime. The patient was always weakest at 6 a.m., and ergograms taken before the 6 a.m. injection, i.e. 9 hr. after prostigmine and 12 hr. after ephedrine, showed complete fatigue with  $\frac{1}{2}$  kg. in less than 2 min. with a total work done of 40–42 kg. cm., a state comparable with the fatigue period of the controls before ephedrine was given. In view of this it is thought that the injections should not have been stopped during the night in this case.

I have to thank Dr H. H. Carleton for permission to study and report this case and his House Physician, Dr C. A. St Hill, for considerable help in taking a number of the ergograms.

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**Synchronous mitoses in a binucleate macrophage *in vitro*.** By F. JACOBY. *From the Department of Physiology, The Medical School, Birmingham*

The mode of formation and the fate of somatic binucleate cells both *in situ* and *in vitro* is still a matter of controversy. Three modes of origin are possible: mitotic or amitotic nuclear division without cell cleavage or fusion of two mononucleate cells. Macklin [1916] advocates the formation via amitosis. If such binucleate cell ever divides mitotically the two nuclei behave like one forming a single mitotic figure and eventually give rise to two mononucleate daughter cells. Lewis [1927], on the other hand, favours the view that mitosis without cytoplasmic division and fusion play at least as great a role in binucleate cell formation as amitosis if not more so. According to this author the mode of formation may depend on the cell "species". He himself observed in living sarcomatous cells the increase in size of multinucleate giant cells by fusion with mononucleate cells or with another giant cell. The fusion of two single cells to form a binucleate cell was not observed. Lewis also describes various modes of "double mitoses" in binucleate mesenchyme cells in which the nuclei were lying more or less side by side.

For both problems (formation and fate of such cells) continuous living observation is indispensable. Evidence derived from fixed material only

is of little value. The following observation, therefore, made on a binucleate macrophage is of general interest and seems worth reporting. The observation was made on a pure culture of "tissue macrophages" living in diluted serum in a Carrel flask. The technique of their isolation from chick embryo heart explants has been communicated previously [Jacoby, 1938]. A part of the culture containing 243 cells was photographed over a long period at 6 min. interval at a magnification of  $150\times$ . On analysing the film an elongated binucleate cell was found the two nuclei of which, lying as it were in "head-tail" position, went simultaneously into mitosis. Cytoplasmic division followed in both equatorial planes. Thus three cells were formed, the two peripheral ones (*a* and *b*) being mononucleate, the

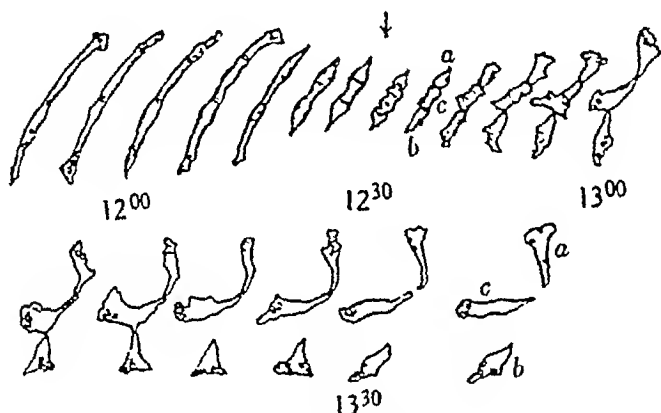


Fig. 1. Tracings taken from 19 consecutive photographs of a binucleate cell undergoing double mitotic division; from culture TM 53/8657/58 b, tissue macrophages, 43 days *in vitro*. Magnification  $150\times$ .

central one (*c*) binucleate. The course of events is shown in Fig. 1. Since first the two and then the four groups of chromosomes were clearly visible ( $\downarrow$ ), in spite of the relatively low magnification, the process could be elucidated with ease. On the actual photographs (paper negatives) the nucleoli and the nuclear outlines, especially prior to division, could also be discerned. The nuclear divisions lasted for about 24–30 min. and it took about an hour from the onset of cytoplasmic division until complete visible separation of the three cells was accomplished. But these cells often remain attached to one another with a thin cytoplasmic process for long periods.

Furthermore, the synchronism of the mitoses is worth noting. Exact synchronization is very rare in tissue cultures both in fibroblastic cell colonies and in pure populations of monocytes or macrophages. Even

bin cells (derived from the mitosis of a single cell) which often remain near each other during the interkinetic period are usually considerably out of step when they enter mitosis. For synchronous mitoses to occur the presence of an organic cytoplasmic continuity seems essential, probably because it establishes identical environmental conditions for the centrosomes and nuclei. This has also been observed in fertilized eggs; if cell cleavage was suppressed experimentally all the nuclei present in the common cytoplasmic unit would divide simultaneously [Polowzow, 1924].

Conclusions: The original binucleate cell must have been formed—not by amitosis, but either by fusion or by mitosis without cytoplasmic division. A binucleate cell can in the above way give rise to further binucleate cells. Such “double mitosis” is only a rare occurrence under the conditions of these experiments. It has only been seen once among more than 3000 mitotic divisions of monocytes and macrophages followed photographically.

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**Negative phase blood produced *in vivo* by trypsin.** By the late J. MELLANBY and C. L. G. PRATT. *From the Department of Physiology, University of Oxford*

It has been shown that negative phase blood is incoagulable because it contains no fibrinogen [Mellanby, 1908]. It may be easily prepared by the intravenous injection of thrombase or thrombokinase in the cat. It may also be produced in the cat by the intravenous injection of a suitable quantity of activated pancreatic juice, and the course of appearance and return of fibrinogen followed by determining the degree of dilution at which the oxalated plasma is still able to gelate when thrombase is added. Normal oxalated cat's plasma will produce a firm clot with thrombase when diluted 40-fold with 0.85% NaCl. If the greatest dilution at which a sample of plasma will clot under the influence of thrombase is 20-fold, that may be taken as evidence that only one-half the normal quantity of fibrinogen is present.

The following experiment illustrates the production of incompletely negative phase blood:

A male cat, 2.7 kg., under chloralose anaesthesia, received 20 ml. activated pancreatic juice intravenously. Before injection, and at intervals after it, 2 ml. samples of blood were removed from the carotid artery, oxalated, and the cells removed by centrifuging. From each sample  $\frac{1}{2}$  ml. plasma was used to set up a series of mixtures of plasma with 0.85 % NaCl containing plasma in the proportions 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32 and 1 : 40 respectively. Thrombase, 0.1 ml., was then added to each tube and the whole incubated. As Table I shows, about four-fifths

TABLE I

Time after injection min.	Maximum dilution in which plasma will clot	Percentage of original fibrinogen remaining
0 (before injection)	1 : 40	100
10	1 : 8	20
30	1 : 4	10
45	1 : 4	10
60	1 : 4	10
120	1 : 4	10
180	1 : 4	10

of the fibrinogen was removed from the circulation within 10 min. and after 30 min. only one-tenth of the original concentration of fibrinogen remained. Replacement of fibrinogen had not begun in 3 hr.

Complete incoagulability may be secured by increasing the dose of trypsin.

A male cat, 2.5 kg., received 25 ml. activated pancreatic juice intravenously. Table II shows the depression and subsequent recovery of fibrinogen concentration.

TABLE II

Time after injection	Maximum dilution in which plasma will clot	Percentage of original fibrinogen remaining
0 (before injection)	1 : 40	10
20 min.	1 : 2	5
30 min.	—	0
5 hr.	—	0
17 hr.	1 : 12	30
25 hr.	1 : 40	100

The rate of recovery is of interest. In the first 17 hr. only about 30 % of the original amount of fibrinogen had been replaced. In the next 8 hr. (or less) the remaining 70 % entered the blood stream.

The rate of reappearance of fibrinogen after its removal from the circulating blood has been studied by Stanbury, Warweg & Amberson [1936] in normal dogs and by Ettledorf, Mitchell & Amberson [1937] in immunized dogs. Using the method of total plasmaphoresis, involving the removal not only of fibrinogen but of all the native proteins of the plasma, both groups of workers appear to have found that replacement of fibrinogen is not complete within 50 hr. The return in less than half that time of the fibrinogen removed by the action of trypsin may indicate either a species difference or that this procedure involves less general depreciation of the physiological condition of the animal or less damage to the source of supply of fibrinogen.

The manner in which trypsin produces negative phase blood *in vivo* is doubtful. Two mechanisms are possible. Either trypsin directly digests fibrinogen, or it liberates thrombokinase, leading to intravascular defibrination of the blood, the fibrin being deposited harmlessly on the endothelium of the small vessels. According to Eagle & Harris [1937] intravenous injection of trypsin in the rabbit rapidly kills the animal, large soft clots being found post mortem in the heart and great vessels, due probably to thrombokinase liberation (though attributed by Eagle & Harris to activation of prothrombase by trypsin). In the cat this does not occur. It is possible that thrombokinase is liberated but that the digestion of prothrombase or thrombase is so rapid that fibrinogen is not clotted. The rapidity with which trypsin digests prothrombase, thrombase and fibrinogen *in vitro* [Mellanby & Pratt, 1938] suggests that negative phase blood *in vivo* is due, not to intravascular coagulation but to fibrinogen digestion.

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**The antikinasic action of cobra venom.** By N. B. MYANT and C. L. G. PRATT. *From the Department of Physiology, University of Oxford*

The anticoagulant action of cobra venom when injected intravenously into dogs and rabbits was shown by Morawitz [1904] to be due to the inhibition of the first phase of coagulation, viz. the conversion of

prothrombase into thrombase by thrombokinase in the presence of calcium salts. This was confirmed *in vitro* by Mellanby [1908] using a solution rich in prothrombase prepared from fowl plasma, but the nature of the antikinasic action remained obscure. The demonstration by Leathes & Mellanby [1939] also *in vitro* that preparations containing lecithine powerfully enhance the action of thrombokinase on prothrombase suggested that cobra venom depresses the activity of thrombokinase by depriving it of the adjuvant influence of lecithine. The venom is known to contain a lecithinase and to convert lecithine into lysolecithine, though to what extent is not certain. Leathes & Pratt [1940] have found that lysolecithine has no adjuvant action on the kinasic action of *Daboia* venom. If the suggestion should prove correct, the antikinasic action of cobra venom *in vivo* would support the important implication that the normal process of the coagulation of the blood involves the action of lecithine as an activator or augmentor of thrombokinase.

Preliminary experiments indicate that the relation between cobra venom and thrombokinase is not a simple one.

(1) *Cobra venom blood*. Cobra venom blood can be prepared by receiving arterial blood from a cat into a vessel containing cobra venom, or more conveniently, by injecting venom 2 mg./kg. intravenously and bleeding the animal within 2 min. (i.e. before the circulation stops), using artificial respiration. The blood so obtained remains fluid at 0° C. for a considerable period of time. After removal of the corpuscles, the following agencies may be shown to bring about coagulation of the plasma:

- (a) *Daboia* venom (a powerful thrombokinase).
- (b) Testis suspension (thrombokinase).
- (c) More cobra venom.
- (d) Trypsin and papain.
- (e) Lecithine.
- (f) Thrombase.

(2) *Activation of prothrombase*. Cobra venom delays the activation of prothrombase by *Daboia* venom in a characteristic way. Although the first indication of activation is detectable almost as soon in the presence of cobra venom as in its absence, the process is not completed with the usual increasing velocity, and the final concentration of thrombase formed is diminished.

The influence of cobra venom on the augmentation by lecithine of the kinasic action of a weak solution of *Daboia* venom depends upon the concentration of the cobra venom. A high concentration abolishes the augmentation whereas a lower concentration enhances it.

concentrations of cobra venom so far tested hasten the activation of thrombase by testis thrombokinase, with and without lecithine. Activation of testis thrombokinase is scarcely augmented by lecithine.

*Proteolytic enzyme.* In common with many snake venoms cobra contains a proteolytic enzyme capable of clotting milk and of giving about the coagulation of fresh fowl plasma just as does trypsin. At concentrations used in these experiments it was not possible to observe a digestive action on thrombase.

*Fowl plasma.* As is the case with trypsin [Mellanby & Pratt, 1938] cobra venom does not clot "ripe" fowl plasma, which is insensitive to the coagulant action of proteolytic enzymes. Such plasma is therefore a convenient test object for the study of the interaction of thrombin and antikinase uncomplicated by interference from the enzyme. Cobra venom diminishes the coagulant action of *Daboia* venom and of testis thrombokinase on "ripe" fowl plasma, with and without added lecithine.

Cobra venom antagonizes in different degrees thrombokinases from different sources. If concentrations of *Daboia* venom and of testis suspension can be selected such that they are equally active towards "ripe" fowl plasma, then the *Daboia* venom coagulates cobra venom plasma more readily than does the testis suspension.

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#### The span of the red blood corpuscles and the maturation time of reticulocytes. By H. S. BAAR and T. W. LLOYD

The duration of life of red cells in the circulation is of physiological and biological importance, especially since attempts have been made to explain the pathogenesis of some anaemic states by estimations of the metabolism of haemoglobin. Investigators have attempted to determine the span of life of erythrocytes by examination of patients receiving blood transfusion, by animal experiment, by observations of the maturation of reticulocytes *in vitro*, and by determination of the

bile pigment excretion. The results have, however, shown an enormous discrepancy. The most notable results have been obtained by transfusion of normal people with blood of the groups *M* and *N*, which suggest that the red cells persist for 55-75 days. Experiments on the maturation time of reticulocytes *in vitro* suggested that the life span was 100 days, and since in this experiment 1% reticulocytes was taken as a normal average, which we consider much too high, about 150 days should be deduced from these findings. From stercobilin excretion 200 days has been calculated, while from some animal experiments a figure of 8-10 days has been reached.

Our investigations include experiments *in vitro*, observation of the rate of maturation of reticulocytes during life, an examination of blood, both by counts and Price-Jones curves, following blood transfusions.

That the red cell normally leaves the bone marrow only in the stage of the reticulocyte appears to be well founded, but observations of discrepancy between blood regeneration and reticulocytosis are not without objection. It may, therefore, be assumed that if the maturation time of the reticulocyte can be determined, it is possible to discover the life of the red cell. All investigations *in vitro* have been made in a solution recommended by Osgood for cultures of bone marrow cells. It seems that sterile precautions, and a solution containing plasma salts in isotonic concentration and sufficient sodium citrate to prevent clotting are the only indispensable conditions. Films are made each from a separate tube at intervals of 2 hr.

The counts show a linear decrease in reticulocytes for the first 4-8 hr.; after 6-8 hr. the slope becomes less steep and may even run a horizontal course for many hours. Simultaneously, morphological changes occur indicating a damage to their vitality. The first few hours only can be considered as an imitation of vital conditions and the true maturation time of the reticulocyte is best obtained by extrapolation of the steep slope to the abscissa. The average time obtained on a series of normal cases, and cases in which there was a slight reticulocytosis was 11 hr. Taking an average 0.7% reticulocytes, a life of 65 days for the circulating red cell is obtained. In cases with high reticulocytosis and many immature reticulocytes the maturation time may be much longer, even 24 hr.; sudden increases in the reticulocyte count are associated with increased maturation time. There is not, however, any fixed relation between the number of reticulocytes and the maturation time, nor do the morphological differences between the Heilmeyer groups allow a calculation to be made from a differential count. In pathological cases



the maturation time must be determined individually. The regeneration rate, a figure indispensable in investigations of haemoglobin metabolism, is calculated by multiplying the reticulocyte percentage by 24 and dividing by the maturation time in hours. Extremely short maturation times, of about 3 hr., have been found in two cases of pernicious anaemia. This may in part explain the discrepancy between the low number of reticulocytes and the high output of coproporphyrin I in this disease.

Determination of the maturation time *in vivo* has as yet been performed once only. A normal person was transfused with blood from an acholuric familial jaundice with considerable reticulocytosis. An immediate rise in the recipient's reticulocytes resulted. Counts at 2 hr. intervals showed a return to the pre-transfusion level in 6-8 hr., a figure frequently encountered in experiments *in vitro*.

The life span of red cells after transfusion was studied in a case of haemolytic anaemia of the newborn. Following repeated transfusions a blockade of the bone marrow resulted and very few reticulocytes appeared in the circulation. It was found by Price-Jones curves that the cells of the patient had been completely replaced by those of the donor. The "aregenerative phase" persisted for 10 days and the drop in the red cell level during this period indicated that the transfused cells would disappear in about 21 days.

After transfusion of normal blood to cases of acholuric jaundice (marked microspherocytosis), and into cases of macrocytic anaemia such as that of icterus gravis neonatorum, and of blood cells showing marked microspherocytosis into normocytic persons, the Price-Jones curve shows the heterogeneity of the red cell population. By Mogesens' method such curves can be "decomposed" into a major and a minor component. The major component is not always identical with the patient's red blood cell population but it is permissible to assume that changes in the number of cells in the major and minor components run parallel to the disappearance of the donor's cells. Such investigations have shown that the normal red cell when transfused into haemolytic anaemias has a short life, but a longer one than that of the patient's own cells, while the pathological red cell of acholuric jaundice disappears very rapidly when transfused into normal persons.

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9 March 1940

**Induction of lactation in goats with diethylstilboestrol dipropionate.** By S. J. FOLLEY, HELEN M. SCOTT WATSON and A. C. BOTTOMLEY. (*From the National Institute for Research in Dairying, University of Reading*)

Numerous workers have shown that oestrogen treatment inhibits lactation [see Folley, 1936], and Nelson [1936] believes that oestrogens play a decisive role in the prevention of lactation prior to parturition. Our experiments indicate that for the ruminant this theory may require modification.

One gram of 1% diethylstilboestrol dipropionate ointment was applied thrice weekly to the udder of a virgin female goat and daily milking begun. After a latent period of 30 days during which a few ml. of fluid were secreted daily, there was a sudden increase in milk yield to a maximum of 1500 ml. daily and then a slow decline. The milk yield curve resembled a normal lactation curve. Omission of the oestrogen from the ointment had no effect on the course of the curve for 20 days, but then there was a sharp fall, not immediately arrested by reapplication of double the original dose of oestrogen. Eventually, especially when the original oestrogen dosage was restored, the milk yield rose once more. The milk secreted during the artificial lactation was normal in composition (nitrogen partition, lactose %) and indeed was of excellent quality (% fat > 6, % non-fatty solids > 10).

Essentially similar results were obtained with two other virgin goats, one of which during a long preliminary control period of daily milking and inunction with base ointment (i.e. ointment without the oestrogen) secreted a small quantity of milk daily, and a virgin heifer, though in the latter case, the maximum yield was small and the secretion never passed the colostrual stage. No milk could be obtained from either the

third goat or the heifer during the preliminary control periods of attempted milking and inunction with base ointment. All attempts to induce lactation in male goats failed even when progesterone was also given.

De Fremery [1938] found that prolactin treatment was necessary to induce artificial lactation in female goats following preparation with oestrogen. These experiments show that oestrogen treatment will cause udder development and copious secretion of normal milk in virgin goats without need for prolactin treatment. Oestrogens at the levels used by us evidently do not inhibit lactation in goats.

We are indebted to Dr F. L. Pyman, F.R.S., and Messrs Boots Pure Drug Co., Ltd., for the diethylstilboestrol dipropionate used in these experiments. Our thanks are also due to Mr C. Kirby for the care of the animals.

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#### **Double representation of the feet in the sensory cortex of the cat.** By E. D. ADRIAN. (*From the Department of Physiology, University of Cambridge*)

The recording of potential changes on the brain surface is now an established method of mapping the receiving areas of the cortex [cf. Kornmüller, 1937]. Recent papers are those of Marshall, Woolsey & Bard [1937] on the sensory area in the monkey and of Bremer & Dow [1939] on the auditory area in the cat. In the course of some experiments on the cat's brain the sensory area has been mapped repeatedly and in one respect the results have been unexpected. The method has been to record the afferent impulses reaching the cortex from different parts of the body in cats anaesthetized with dial or chloralose. The sensory receiving area determined in this way (Fig. 1) agrees very well with the sensory field shown in Campbell's map of the cat's brain [Campbell, 1905]. In the anterior part of the area (Fig. 2) the localization follows the expected arrangement. The main region for the trunk and limbs is in the posterior sigmoid gyrus immediately behind the motor area: impulses from the hind limbs arrive in the cortex very close to the mid-line; the trunk, shoulder, elbow and forefoot regions extend laterally to

the coronal sulcus and the regions for neck, ear, face, lips and snout follow on in the anterior part of the suprasylvian gyrus. But finally, behind the head area and in the ectosylvian gyrus there is a second region for the forefoot and a small one behind it for the hindfoot. These give a maximal response to touches on the claws and scarcely any to touches above the wrist or ankle. The area for the hindfoot is sometimes

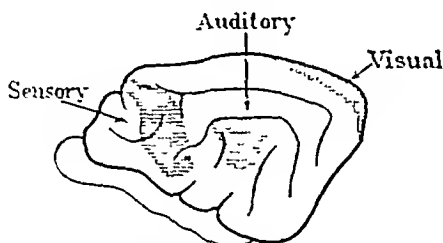


Fig. 1. Extent of the sensory receiving area in the cat's brain

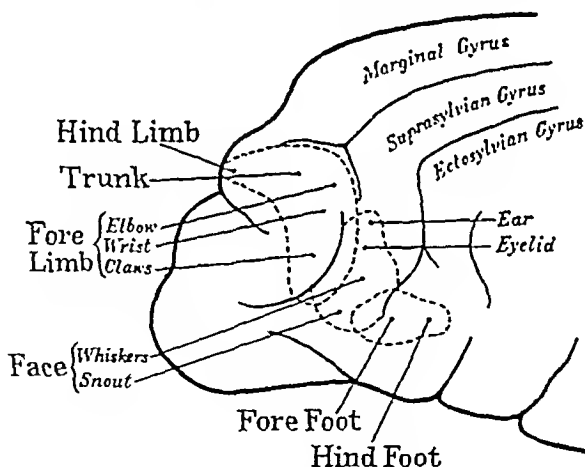


Fig. 2. Localization within the sensory receiving area, showing the double representation of the feet.

not more than 1 mm. square, but it has always been found caudal to the forefoot area, so that the two regions for the hindfoot may be separated by as much as 2 cm. though the two regions for the forefoot are closer together.

The meaning of this double representation of the feet is uncertain, but it is not found in the dog and so may well be related to the special movement of the claws in the Felidae. The boundaries of the posterior foot area lie near the point marked by Ferrier [1886] as responding to

stimulation by giving a clutching action of the paw with protrusion of the claws and this motor response often follows stimulation of the area by weak shocks. The area also corresponds in position with Tower's "lateral extrapyramidal motor area" [Tower, 1936].

The map in Fig. 2 shows the termination of the most direct pathways by which afferent impulses can reach the cortex. It gives the arrival areas as they are found in deep anaesthesia, when only the opposite side of the body is represented and the localization is stable and sharply defined. In lighter anaesthesia there is more irradiation: an abrupt tap on any of the feet may give a widespread response [cf. Forbes & Morison, 1939] and the primary afferent discharge is followed by an oscillating after-discharge of impulses from the thalamus. The points of maximum response remain unaltered, but clearly there is no reason to expect complete agreement between this map and maps of the sensory area as defined in some other way, e.g. by Dusser de Barenne's strychnine method.

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# PROCEEDINGS

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# PHYSIOLOGICAL SOCIETY

### 4 May 1940

#### The diffusion of large molecules through the cell membrane.

By J. F. DANIELLI. *From the Biochemical Laboratory, Cambridge*

For molecules of, for example, glycerol, penetrating ox erythrocytes, the resistance to free diffusion lies almost entirely at the membrane oil-water interface [Danielli, 1939], and is due to the high potential energy barrier restricting diffusion from water to oil. This potential energy barrier is mainly due to "hydrogen" bonds formed with water by each polar group of the diffusing molecule. All these bonds must be broken simultaneously before penetration of the oil layer occurs.

In the case of proteins (e.g. ovalbumin) each molecule contains many polar groups and forms many "hydrogen" bonds with water. For proteins forming only 100 bonds the minimum kinetic energy for penetration is 100,000 cal., which gives a maximum permeability of one molecule per  $\text{cm}^2$  in  $10^{150}$  years per mol. per litre concentration difference. Alternatively, one may assume that the protein penetrates by knocking a cylindrical hole in the membrane. Then the minimum possible kinetic energy for penetration is equal to the free surface energy of the hole and removed cylinder. This gives a minimum rate of one molecule per  $\text{cm}^2$  in  $10^{32}$  years. Thus it appears that proteins cannot penetrate a thin oil layer by ordinary diffusion processes, and that where penetration of a cell membrane occurs, it must be through some specialized area.

But large fatty molecules form few "hydrogen" bonds and will readily penetrate into the oil layer, and the chief resistance to penetration will arise in moving out of the membrane into water. The minimum possible energy required for this is 500–1000 cal. per  $\text{CH}_2$  group, giving a maximum rate for oleic acid of  $10^{-2}$  to  $10^{-5}$  g. mol.  $\text{cm}^2/\text{day}$ . The maximum rate of penetration of the intestinal villi is about  $10^{-4}$  g. mol./ $\text{cm}^2/\text{day}$ , so that fatty acids may penetrate by a simple diffusion process. But with tristearin the calculated maximum possible rate is one molecule in a year

per cm.<sup>2</sup> This is too small to be of practical importance, so that if triglycerides penetrate a cell membrane they must do so by some special mechanism. Sterols fall into an intermediate group concerning which no decision can be reached from the calculations.

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**The ageing of fowl plasma.** By C. L. G. PRATT. *From the University Laboratory of Physiology, Oxford*

Fresh stable fowl plasma is coagulated in a characteristic manner by the addition to it of trypsin. This sensitivity to trypsin gradually declines and ultimately disappears when the plasma is kept for several weeks at 0° C., and at the same time a scanty white precipitate is deposited. By digesting the precipitate with activated pancreatic juice it is possible to prepare a solution which will bring about coagulation of the "ripe" supernatant plasma. These observations were made by Mellanby & Pratt [1938], who put forward the suggestion, in order to account for them, that the process of ageing consists essentially in the deposition of a hidden thrombokinase which in fresh plasma can be liberated by trypsin. However, the observation of Leathes & Mellanby [1939] that the action of thrombokinase on prothrombase in the presence of calcium can be considerably accelerated by lecithine suggests that a factor in the ageing of fowl plasma may be the inactivation or precipitation of lecithine or of a substance which can be replaced by lecithine. The following facts support this view:

(1) "Ripe" fowl plasma can be coagulated by trypsin if lecithine be first added to it.

(2) As the plasma ages, its resistance to coagulation by the thrombokinase of *Daboia* venom increases considerably, whereas its resistance to coagulation by a watery suspension of testis is only slightly increased. Now the action of *Daboia* venom upon prothrombase is greatly assisted by lecithine; that of testis thrombokinase is not.

(3) The resistance (as indicated by the clotting time) of "ripe" plasma to *Daboia* venom is greatly diminished by the addition of lecithine, but the resistance to testis thrombokinase is scarcely lessened by this means.

If the phenomenon of the ageing of fowl plasma is due to the progressive removal of lecithine, then it is probable that lecithine (or a substance closely associated with it) plays a part in the normal process of blood coagulation.

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**Lymphocytosis induced by extracts from lymph nodes.** By H. L. MEYERHOF. *From the Research Department of the Istituto Sieroterapico Milanese, the Institute for Microbiology, University of Milan, and the Department of Pathology, Cambridge*

The results hitherto obtained on the effect of extracts from lymph nodes on the lymphocyte count are conflicting. We have found that the mesenteric lymph nodes of the calf contain a principle which induces lymphocytosis in the guinea-pig. The glands extracted contained 74-80 % water; 14.8-17.2 % of the dry weight were lipins. The active principle was soluble in alcohol [confirming Rocchini, 1937] and insoluble in acetone. Water or saline did not extract the active principle from fresh or dried glands. The usual extracts were prepared by alcohol extraction of the acetone insoluble residue (the phosphatide fraction, which contained 54.4 % of the lipins) and were taken up in *aqua dist.* When suspended in olive oil, however, the extracts were inactive. Extracts similarly prepared from other organs (heart, kidney, liver) of the calf produced no lymphocytosis, even if taken up in *aqua dist.*

The following fractions were inactive: (1) alcohol insoluble, ether soluble; (2) alcohol and ether insoluble, chloroform soluble; (3) acetone and alcohol soluble; (4) acetone soluble, alcohol insoluble; (5) acetone and alcohol insoluble, ether soluble; (6) acetone, alcohol and ether insoluble, chloroform soluble.

Daily subcutaneous injections (0.5-3.0 c.c.) were given over a period of 3 weeks or longer. 1 c.c. contained the equivalent of 0.4-17.3 g. of fresh tissue. The lymphocytes in the peripheral blood started to increase 10-20 days after the beginning of the injections. After 30 days the average increase was about 200 %. The highest increase observed was about 640 %. When the injections were discontinued the lymphocytes returned to their normal number within a month. The number of the



other white cells remained unaffected. There were no hyperplastic changes in the lymphatic system.

Amounts of extracts which were active when injected into guinea-pigs had no effect on the number of lymphocytes in the blood when injected intramuscularly into human beings. Larger amounts have not been examined.

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#### **The stimulating effect of glucose and pyruvate on the rabbit's gut.** By W. FELDBERG and O. M. SOLANDT. *From the Department of Physiology, University of Cambridge*

Glucose or pyruvate is necessary for the liberation of acetylcholine in perfused stimulated sympathetic ganglia [Kahlon & MacIntosh, 1939] and accelerates synthesis of acetylcholine in brain tissue [Mann, Tennenbaum & Quastel, 1938; see however Stedman & Stedman, 1939]. We have tried to find out if the stimulating effect of glucose and pyruvate on the isolated rabbit's gut can be similarly explained.

A piece of small intestine suspended in glucose-free Tyrode's solution relaxes and the rhythmic contractions diminish and may disappear. Addition of 5-50 mg. of glucose or 2-20 mg. of pyruvate to the bath (14 c.c.) has, after a latency of 8-40 sec., a stimulating action. The smaller doses mainly increase the amplitude of rhythmic contraction, the larger ones greatly increase tone. Phloridzin inhibits the stimulating action of glucose and to a lesser extent that of pyruvate.

About 100 cm. of the gut were perfused from the artery at a rate of about 2.5 c.c. per min. with eserinizied Locke's solution. The outflowing fluid was collected from a cannula in the vein and from the lumen. In three experiments with glucose 1.5, 3.5 and 4.5  $\mu$ g. of acetylcholine and 1.5, 1.5 and 2.1 mg. of choline were obtained from both sources in 1 hr. The corresponding figures for three experiments with glucose-free solution were 1.8, 5.5 and 5.2  $\mu$ g. and 1.8, 1.4 and 2.9 mg.

Magnus [1930] suggested that the stimulating action of pyruvate on the gut might be due to formation of a choline ester. Rona & Neukirch [1912a, b] attributed the activity of glucose and pyruvate mainly to their action on the metabolism of smooth muscle. Our results support the latter view and indicate that the stimulating effects of glucose and

pyruvate on the isolated gut do not result from synthesis or increased liberation of choline or acetylcholine. This conclusion is supported by the fact that an acetylcholine contraction of the gut in glucose-free solution is not sustained and that glucose and pyruvate have a stimulating action on the muscle of the isolated rabbit heart where acetylcholine would have an inhibitory effect [Rona & Neukirch, 1912c].

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**The photoelectric determination of blood CO.** By D. K. HILL  
 (introduced by J. McMICHAEL). *From the British Postgraduate Medical School*

Blood-volume determinations by the CO method are performed with no detriment, even to moderately anaemic patients, if about 10 % of the total haemoglobin is put out of action. The accuracy of the method then depends almost entirely upon the accuracy with which the percentage of carboxyhaemoglobin in the blood can be measured. Certain investigations in relation to traumatic shock require determinations of *circulating* blood volume which can be relied upon to  $\pm 5$  % and it is therefore necessary to estimate the percentage of carboxyhaemoglobin to  $\pm \frac{1}{2}$  %. This can be accomplished by manometric methods, but the technique is time-consuming and difficult of rapid repetition on large numbers of cases. The demonstration is of an alternative "physical" method depending on the difference in light absorption by oxy- and carboxyhaemoglobin, and has the advantages of being quicker and simpler. The blood can be taken from a finger prick and this avoids venesection which in certain subjects may give difficulty. The method has been described by Hartmann [1937] and by Steinmann [1938].

At the wave-length of the green band of the mercury vapour lamp (546 m $\mu$ ) oxyhaemoglobin and carboxyhaemoglobin have the same specific absorption, and at the wave-length of the yellow band (577-579 m $\mu$ ) their absorptions differ markedly. The absorption of the blood (diluted

l in 100) is therefore found at these two wave-lengths: the total haemoglobin and percentage saturation with carbon monoxide can then be calculated. Light intensities are measured by vacuum photocells, the recording instrument being a Lindemann electrometer.

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# PROCEEDINGS

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# PHYSIOLOGICAL SOCIETY

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**Adrenaline ester.** By DEREK RICHTER (introduced by Prof. S. NEVIN). *From the Central Pathological Laboratory, West Park Hospital, Epsom*

In considering the fate of adrenaline in the animal body it has generally been assumed that adrenaline is oxidized in the tissues. Experiments *in vitro* have shown that adrenaline may be oxidized in a number of different ways: it is oxidized to adrenochrome by (a) catechol oxidase [Green & Richter, 1937], (b) the cytochrome system, (c) peroxidase, and (d) pseudophenolases such as the copper-protein complexes [Bhagvat & Richter, 1938], while it is oxidized in the side-chain by the amine oxidase [Blaschko, Richter & Schlossmann, 1937; Richter, 1937]; but there is no evidence that adrenaline is oxidized *in vivo*.

It has now been found that after administering adrenaline (0.13–0.4 mg./kg. by mouth) in man a substance appears in the urine which has the properties of an adrenaline ester; it is readily hydrolysed by heating with acid to give adrenaline. The amount found in the urine was estimated as equivalent to 70% of the amount of adrenaline administered.

The precise physiological significance of this adrenaline ester is not yet clear: it may be an inactivation product or may be primarily concerned in the physiological action of adrenaline; but the evidence at present available shows that esterification, and not oxidation, is the main fate of adrenaline administered under these conditions *in vivo*.

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**So-called negative intrapleural pressure.** By D. BURNS. *From the Medical School, Newcastle-upon-Tyne, University of Durham*

There are two common explanations of the fact that during health the parietal and visceral pleura are in close apposition. Many text-books state that the tendency of the two surfaces to separate on account of the elasticity of the lungs is opposed by "the atmospheric pressure which bears only on the inner surface", "The lungs shrink when atmospheric pressure is equalized on the outside and inside of the sacs". While the latter statement is true it is a misleading though apparent confirmation of the previous hypothesis. This can easily be disproved by reducing the air pressure within the lungs to a value well below the "negative intrapleural pressure" without collapse. Another explanation vaguely mentions "hydraulic traction" exerted on one pleural surface by the other. This was the view of West who demonstrated that the surface tension of water was adequate to prevent two disks covered with pieces of stretched stomach wall from being pulled straight asunder. Emerson points out that under such conditions it takes almost as much force to cause them to glide freely over one another as to pull them apart. I am indebted to Dr J. A. Saunders for a simple model with which it is easy to demonstrate that pressure in the "lungs" can be reduced to a value far below the lowest intrapleural pressure without any collapse of the "lungs" even if capillary active substances are introduced into the "pleural sac". The pleural surfaces remain in apposition because the force tending to cause separation of the surfaces is minute compared with the tensile strength of water. A film of water at body temperature can sustain a tension of over 3600 mm. Hg to the sq. cm. without rupture. The introduction of gas into the pleural sac allows shear to take place but, if the amount of gas is limited, it is soon absorbed and the lungs expand again. Experimentally [West and others] rupture of lung tissue takes place before suction applied through the trachea has any separating effect on the pleural surfaces.

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**The effect of parathyroid extract on gastric secretions.** By F. J. ELLIOTT. *From the Medical School, Newcastle-upon-Tyne, University of Durham*

Lennox [1933] showed that a fall in free and combined HCl, and in peptic activity of the gastric juice followed parathyroidectomy in dogs. Administration of calcium salts restored normality.

The injection of 50-75 units of parathyroid extract ("Euparatone") to normal dogs markedly raises acidity (total and free) and peptic activity. This increased gastric activity continued as long as the blood calcium level was above normal and fell as the blood calcium became lowered.

*A typical example of the results obtained*

Dog no. 2. 10 months old, weight 9.8 kg. Serum calcium 9.5 mg./100 ml.

Sample	Free HCl*	Total acidity*	Pep.int†
(1) $\frac{1}{2}$ hr.	—	0.5	1
(2) 1 hr.	0.3	2.2	4
(3) $1\frac{1}{2}$ hr.	0.4	2.0	12
(4) 2 hr.	—	1.8	6

After injection of 75 units of "Euparatone". Serum calcium 12.7 mg./100 ml.

Sample	Free HCl*	Total acidity*	Pep.int†
(1) $\frac{1}{2}$ hr.	—	2.7	14
(2) 1 hr.	2.8	5.6	20
(3) $1\frac{1}{2}$ hr.	3.2	5.8	2
(4) 2 hr.	1.8	4.0	1

\* Figures given in ml. N/10 NaOH required to neutralize 10 ml. sample.

† These figures are comparative and are obtained by taking the amount of cdestin digested by various dilutions of the gastric juice under constant conditions.

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**Temperature acclimatization in amphibia.** By K. MELLANBY (introduced by G. A. CLARK). *From the Sorby Research Laboratory, University of Sheffield*

I showed recently [Mellanby, 1939] that the behaviour of certain insects at low temperatures was greatly affected by the conditions of exposure during the 24 hr. previous to the experiment. Those kept at high temperatures (e.g. 30° C.) were much more easily immobilized by cold and killed by temperatures below zero than those from cooler

conditions (10–15° C.), these latter having benefited by the cooler acclimatizing conditions.

Amphibia (the frog, *Rana temporaria* L. and the spotted salamander, *Salamandra salamandra* L.) behave in a similar manner. A frog or a salamander kept for 24 hr. at 10° C. is fully active and remains active for days when put in water at 0° C., whereas one kept for 24 hr. at 30° C. is almost completely immobilized at 0° C. Both animals assume a body temperature approximating to that of their surroundings within 15 min. It is possible to take one frog or salamander and acclimatize it to 30° C. when it will be immobilized at 0° C., then keep it at 10° C. for 24 hr. after which it will be active when cooled to 0° C., and then repeatedly transfer the animal from one temperature to another and observe repeated acclimatization. It should be noted that no acclimatization takes place if an animal from 10° C. is warmed to 30° C. for 30 min. (internal body temperature) and immediately cooled to 0° C.; it behaves as though transferred directly from 10° C. The similarity of the behaviour of amphibia and insects makes me suspect that this is a widespread phenomenon in which temperature may alter the properties of the general protoplasm of the animals. Not only is the general behaviour of the animal affected but various organs give characteristic responses. Thus at 0° C. a "10° Salamander" has a heart rate of approximately 20 beats per minute, while at the same low temperature a "30° C. salamander's" heart beats 8 times or less in a minute. These characteristic rates are maintained at this temperature for many hours, both in pithed and in intact animals. Though the two species of amphibia behave in essentially the same manner, there are differences in the rate of acclimatization and the extent to which these changes occur.

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#### The potential difference accompanying a wave of activity.

By H. E. ROAF. *From the Department of Physiology, University of Liverpool*

When a tissue becomes active the active portion becomes negative compared with its resting state. Craib [1930] has recorded potentials showing that triphasic changes may be obtained when a wave of activity passes an electrode. The explanation of these triphasic changes is

probably as follows: When a tissue becomes active its surface becomes permeable and the negativity of its interior, due to the Helmholtz double layer, can be recorded from the surface contrasted with the relative positive charge on the resting surface.

When negative electrons escape from the interior, it becomes less negative, i.e. more positive. As the charges are attached to ions the finite speed of their movement may limit the increased positivity in the interior to parts of the tissue near the permeable portion. The Helmholtz double layer, if it maintains a constant difference in potential between the inside and outside of the tissue, will cause a relative increase in positive charge at that part of the surface covering the increased positivity inside, i.e. adjacent to the zone in which the surface has become permeable.

In Craib's [1930] experiments, the distal electrode being at a distance from the tissue, any small difference in potential at it will not show much change as a wave of activity passes along the tissue, but the electrode touching the tissue would show localized changes in potential of which the explanation is given above. Hodgkin & Huxley [1939] with an electrode inside the nerve showed the effects of stimulation of the giant nerve of squid. They published a record in which the marked negative deflexion is accompanied by a positive charge such as would be expected in the theoretical description given above.

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#### **Triphasic electrical changes.** By H. E. ROAF. *From the Department of Physiology, University of Liverpool*

Craib [1930] and others [Eccles & O'Connor, 1939] have stated that triphasic electrical changes are due to the active tissue being immersed in a large volume of electrolyte. This is not the only way in which triphasic changes can be obtained and Hoffmann [1912] has already shown such changes with muscle suspended in air. Three recording electrodes are used with the middle one linked to one lead of the recording system and the two outer electrodes linked through equal resistances with the other lead. When a wave of activity passes the electrodes three



potential changes will be recorded: (1) when the activity reaches electrode 1 it will cause a deflexion showing half the potential between electrodes 1 and 3: (2) when the activity reaches electrode 2 it will cause a deflexion in the opposite direction but of the full value of the potential established under electrode 2: (3) when the activity reaches electrode 3 it will cause a deflexion in the same direction as (1) and again half the



Fig. 1. Triphasic response from frog's sartorius. From above downwards: electrical response, time marking in 100 D.V. per sec. and signal for point of stimulation. Note stimulation artefact at time of stimulation followed by triphasic change (up, down, up); the middle change is larger than the other two because it is full potential at electrode 2. The other two are smaller because they represent half the potential between electrodes 1 and 3.

potential difference between electrodes 1 and 3 because the lead to the recording system has equal resistances on each side of it between electrodes 1 and 3 respectively.

Therefore triphasic electrical responses can be obtained by tissues suspended in air. It does not necessarily follow that Craib's distal electrode corresponds to the lead united by equal resistances to electrodes 1 and 3.

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